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DISTURBANCE OF THE PITUITARY-ADRENAL INTERRELATIONSHIP IN DISEASES OF THE CENTRAL NERVOUS SYSTEM*

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ABSTRACT

Of 14 patients with disorders of the hypothalamus or temporal lobes, 3 showed a disturbance of the reciprocal pituitary-adrenal interrelationship. These 3 patients failed to manifest evidence of increased pituitary ACTH secretion in response to lowered levels of plasma cortisol, as judged by the inability of the 11β hydroxylase inhibitor, Su-4885, to elicit the expected increase in urinary 17-KGS excretion. In these 3 patients there was no radiologic evidence of pituitary disease; the baseline excretion of 17-KGS was normal, and the adrenal response to intravenous ACTH was normal. Two of the 3 patients were tested with large doses of dexamethasone and failed to show the expected fall in urinary 17-KGS or 17-KS. It appears probable that the basic neurologic disorder in these patients had disrupted the normal function of

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certain central nervous system centers sensitive to varying levels of circulating glucocorticoids and controlling the modulation of pituitary ACTH secretion. One patient unresponsive to both Su-4885 and dexamethasone reacted to the intravenous injection of bacterial pyrogen with a prompt rise in the level of plasma 17-OH-CS and urinary excretion of 17-KGS and 17-KS. Moreover, the normal diurnal variation in plasma 17-OH-CS was found in the 2 patients who were unresponsive to Su-4885. It is thus possible that the mechanisms leading to the normal diurnal variation of plasma 17-OH-CS and to the normal adrenal response to stressful stimuli do not involve those centers postulated to control the reciprocal pituitary-adrenal interrelationship.

IN THE absence of stressful stimuli, the secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary appears to be inversely related to the level of circulating glucocorticoids (1, 2). Whether this reciprocal relationship involves the direct action of glucocorticoids on the anterior pituitary or whether the effect is mediated by the central nervous system has been a point of controversy. The original observations of deGroot and Harris (3) and Hume and Wittenstein (4) as well as the subsequent work of other investigators in the past decade have clearly established that the anterior pituitary is, at least partially, under the control of the hypothalamus. Mason (5) has also emphasized the importance of various components of the limbic system in the control of pituitary ACTH secretion. The clinical occurrence of gross endocrine deficits in association with central nervous system disease (6–10) suggests that in man, as well as in experimental animals, the anterior pituitary is under neural control.

If indeed the central nervous system (CNS) plays an important role in the reciprocal adrenal-pituitary interrelationship, then it is conceivable that certain patients, with strategically located CNS lesions, should fail to exhibit the normal pituitary response to elevated or depressed levels of circulating glucocorticoids. The present study was designed to evaluate by indirect means the pituitary response to altered levels of glucocorticoids in patients with disease of the hypothalamus or temporal lobes. Of 14 patients studied, 3 were found in whom the secretion of ACTH appeared to be relatively independent of changing levels of circulating glucocorticoids. These 3 patients excreted normal quantities of urinary 17-ketogenic steroids (17-KGS) and manifested no clinical evidence of adrenal insufficiency. One of these patients also showed a normal adrenal response to the intravenous injection of bacterial pyrogen.

MATERIALS AND METHODS

Clinical. Fourteen patients with disease of the hypothalamus or temporal lobes were studied (Table 1). All had clinically normal glucocorticoid function. The excretion of 17-ketosteroids (17-KS) was low in Patients P.vT., J.K., K.Mc. and T.S., and the excretion of 17-KGS was borderline low in Patients J.K. and T.S. The low steroid excretion

in these instances may have been due to the nonspecific depression of steroid excretion observed in chronic illness. The hypogonadal state of Patient P.vT. may also have contributed somewhat to the depression of urinary 17-KS. Direct pituitary involvement by the basic disease process appeared unlikely in all patients except N.A., A.T. and A.Th., in whom primary pituitary tumors could not be ruled out. Patients D.O., W.N., J.K., K.Mc., C.F. and T.S. were taking diphenylhydantoin (Dilantin) at the time of this study.

Su-4885 test. The pituitary response to lowered levels of circulating cortisol was assessed by means of an inhibitor of 11β hydroxylation (11, 12), Su-4885 (2-methyl-1,2 bis-(3 pyridil)-1-propanone). In normal subjects, the increased secretion of pituitary ACTH in response to the lowered level of plasma cortisol is manifested by an enhanced excretion of urinary 17-KGS.

The Su-4885 test was conducted over a period of three days. On the first day, a baseline excretion of 17-KGS was determined. On the second day, the drug was administered in a dosage of 750 mg. every four hours by mouth. The urinary excretion on the third day was then compared to the control value determined on the first day. Generally, the maximal excretion of 17-KGS occurs on the third day of the test, although Patient J.K. in the present series was exceptional insofar as she showed a maximal rise on the second day of the test. A patient who failed to show a 20 per cent rise in 17-KGS was considered to be unresponsive to Su-4885.

Dexamethasone suppression test. Dexamethasone, a potent synthetic glucocorticoid, can suppress pituitary ACTH secretion in normal subjects without significantly contributing to the level of urinary 17-KGS (13). The effect of the CNS on the pituitary response to elevated levels of glucocorticoids was assessed in 4 patients (D.O., W.N., S.M. and N.A.). Dexamethasone was administered in the dosage schedules indicated in Figure 2. The 24-hour excretions of 17-KGS and 17-KS on the fourth day of dexamethasone treatment were compared to control values obtained on the day prior to the administration of the drug.

Other tests of pituitary-adrenal function. A standard intravenous ACTH test was performed in 7 patients (14). The diurnal variations of plasma 17-hydroxycorticosteroids (17-OH-CS) (15, 16) were determined in 9 patients on the basis of serial specimens between 9 a.m. and 4 p.m. The adrenal response to the infusion of corticotropin-releasing factor (CRF, "fraction D" of Guillemin) and to the intravenous injection of bacterial pyrogen was studied in 1 patient (W.N.) of special interest.

Biochemical methods. 17-KGS were determined by the method of Norymberski after initial borohydrate reduction of 17-KS (17); the normal 24-hour range in this laboratory is 7–25 mg. for males and 6–22 mg. for females. 17-KS were measured by the method of Drekter *et al.* (18); the normal 24-hour range is 6–22 mg. for males and 6–15 mg. for females. Unconjugated plasma 17-OH-CS were determined by a modification of the method of Peterson *et al.* (19); the normal resting range is 3–24 μg. per 100 ml., in both sexes, and after a four-hour infusion of 25 mg. of ACTH the level rises to between 25 and 50 μg. per 100 ml. Paper chromatography of urinary ethyl acetate extracts after enzymatic hydrolysis was carried out in a Bush B_5 system (20), as previously described (21).

RESULTS

Su-4885 tests

Table 1 includes the results of all Su-4885 tests performed in this study. The data are graphically represented in Figure 1, which also contains pre-

TABLE 1. SUMMARY OF STUDIES OF ADRENOCORTICAL FUNCTION IN PATIENTS WITH CEREBRAL DISEASE

Patient		Diagnosis	Endocrine status	SU-4885 test, Urinary 17-KGS (mg./24 hrs.)			Baseline urinary 17-KS (mg./24 hrs.)	Diurnal variation of plasma 17-OH-CS	Dexamethasone suppression test	ACTH test, plasma 17-OH-CS (μg./100 ml.)	
Name	Age (yrs.) & sex			Day 1	Day 2	Day 3				before ACTH	after ACTH
				Not Responsive to SU-4885							
P.vT.	18, M	Tumor of third ventricle	Sexual retardation; no gonadotropins	17	15	16	3	Normal	—	20	43
D.O.	9, F	Cerebral atrophy; convulsive disorder	Precocious puberty	8	—	8	9	—	No suppression	9	29
W.N.*	45, M	Post-traumatic temporal lobe epilepsy	Normal	20 22	22 24	22 20	23	Normal	No suppression	19	40
				Responsive to SU-4885							
P.E.	17, F	Tumor of third ventricle	Amenorrhea; no gonadotropins	8 12	— —	102 35	6	Normal	—	30	58
J.K.	56, F	Suprasellar cyst	? Hypothyroidism	6	51	22	2	Normal	—	20	36
N.A.	63, F	Suprasellar tumor	Normal	22 15	— —	25 54**	10	Normal	Suppression**	29	57
K.Mc.	30, F	Tumor of septum pellucidum	Normal	14	26	31	2	—	—	—	—
C.F.	20, F	Temporal lobe epilepsy	Normal	12	18	20	7	Normal	—	—	—
J.M.	50, M	Temporal lobe epilespy	Normal	12	14	87	11	Normal	—	—	—
T.S.	55, M	Temporal lobe epilepsy	Normal	6	12	19	5	None†	—	—	—
G.S.	37, F	Meningioma sphenoid ridge	Normal	10	23	32	11	—	—	—	—
S.M.	30, M	Pinealoma	Diabetes insipidus	15	16	27	14	Normal	Suppression	17	40
A.T.	50, F	Suprasellar tumor	Hypothyroidism; diabetes mellitus	13	31	72	10	—	—	—	—
A.Th.	50, F	CNS disease of unknown etiology	Hypothyroidism; diabetes insipidus	22	29	39	13	—	—	—	—

* Special studies of pituitary responsiveness in this patient are summarized in Figure 3 and Table 2.
** Dexamethasone suppression test performed after Su-4885 Test #2, when patient had regained responsiveness to Su-4885 (see text).
† Level at 7:30 a.m., 17 μg.; at 2:00 p.m., 21 μg.; and at 6:00 p.m., 17μg. per 100 ml.

viously published results of Su-4885 tests in 5 normal subjects (22). A greater degree of variation in the response to Su-4885 was evident among the patients with cerebral disease than among the 5 normal controls. In some measure, this variation may have been related to the physiologic setting of the test. For instance, two separate tests were performed on Patient P.E. The first, carried out in the immediate postoperative period, elicited a dramatic rise in urinary 17-KGS; the second, performed two months later when the patient was clinically greatly improved, elicited a more normal response. Another patient, N.A., exhibited no response to Su-4885 at the beginning of a course of x-ray therapy to the hypothalamic region but showed

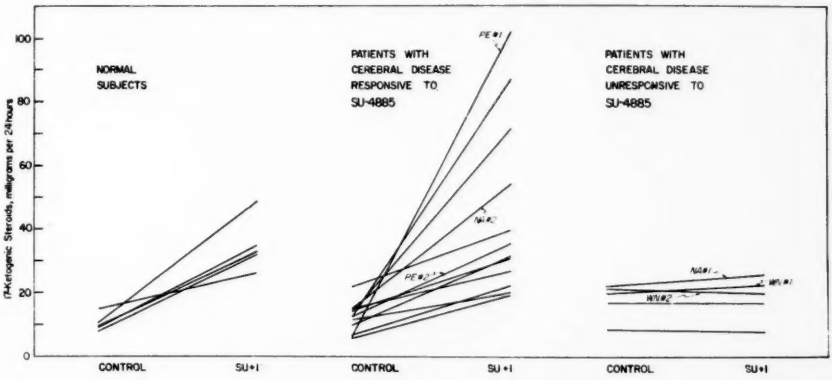

FIG. 1. Su-4885 tests in normal subjects and in patients with cerebral disease. "Control": 17-KGS excretion on the day before administration of Su-4885. "Su+1": 17-KGS excretion on the day following administration of Su-4885.

a normal rise in 17-KGS when the test was repeated at the end of the three-week course of radiation.

Three patients failed to respond to Su-4885 at times when they were under no unusual stress and not subject to radiation therapy. They did not appear chronically ill or debilitated. Two of the 3 patients received Su-4885 in the usual dosage schedule (750 mg. every four hours), but Patient D.O., a 9-year-old child, received 250 mg. every four hours. The adequacy of this dosage in inhibiting adrenal 11β hydroxylation was verified by the results of paper chromatography of urinary extracts obtained on each of the three days of this test. Administration of Su-4885 caused the expected decline in the excretion of tetrahydro-E and tetrahydro-F with a concomitant increase in the level of tetrahydro-S. Similar chromatographic studies with analogous results were performed in association with each of the two Su-4885 tests conducted on Patient W.N.

Because of the unusual interest of these 3 patients, their clinical histories are briefly presented.

P.vT., an 18-year-old white male, had undergone neurosurgical exploration for a tumor of the third ventricle five years previously. No pituitary encroachment by the tumor was noted. Postoperatively, he was given a course of radiation treatment. Three years prior to the study he noted increasing obesity. Because of failure of normal sexual maturation, he was treated with 5–15 mg. of methyltestosterone per day. This medication was stopped two weeks before the start of the present studies. Physical examination revealed obesity (257 pounds), small testes and penis, and a paucity of body and facial hair. Laboratory studies: BMR, −29 per cent; 24-hour thyroidal I^{131} uptake, 19 per cent; serum cholesterol level, 244 mg. per 100 ml.; urinary gonadotropin titers negative at 5 and 10 units; urine specific gravity, 1.030. The patient was not taking any medication at the time of these studies.

D.O., a 9-year-old white girl, had a life-long history of convulsions (both the grand mal and the temporal lobe variety), mental retardation, and precocious puberty since the age of 8 months. Pneumoencephalograms showed generalized ventricular dilatation suggestive of cerebral atrophy. She had no symptoms or signs indicating any abnormality in adrenal, thyroid or posterior pituitary function. Physical examination showed precocious breast and genital development. Laboratory studies: urinary gonadotropin titers positive at 5 mouse uterine units; serum cholesterol level, 145 mg. per 100 ml.; 24-hour thyroidal I^{131} uptake, 24 per cent; and serum PBI level, 5.4 μg. per 100 ml. The patient was taking diphenylhydantoin, phenobarbital, and trimethadione at the time of these studies.

W.N., a 43-year-old white male, had a history of temporal lobe epilepsy following head injuries in an automobile accident five years prior to the study. There were no symptoms suggestive of any endocrine abnormalities, except a history of polydipsia. However, the highest urinary specific gravity in random samples was 1.020. Results of the Hickey-Hare test were normal. There was no evidence of diabetes mellitus or primary renal disease to account for the large water exchange (3–5 liters per day). Other laboratory studies included: serum PBI level, 3.8, 4.5 and 3.3 μg. per 100 ml.; and BMR, −21 and +26 per cent. The patient was taking diphenylhydantoin and phenobarbital at the time of these studies. The tendency toward low serum PBI values was attributed to the action of diphenylhydantoin, which has been shown to interfere with the binding of thyroxine to plasma proteins without changing the metabolic status of the patient (23).

These 3 patients had the following similarities: 1) none showed any radiologic evidence of pituitary disease; 2) their histories did not suggest adrenal insufficiency; 3) all had normal baseline levels of urinary 17-KGS; and 4) all showed a normal adrenal response to the infusion of ACTH.

Dexamethasone suppression tests (Fig. 2)

Four dexamethasone supression tests were performed, 2 in patients (D.O. and W.N.) unresponsive to Su-4885 and 2 in patients (S.M., and N.A. after the completion of x-ray therapy) normally responsive to Su-4885. Dexamethasone suppressed urinary 17-KGS and 17-KS in the patients normally

responsive to Su-4885 but failed to do so in the patients unresponsive to Su-4885.

Special studies of pituitary responsiveness

Additional studies were performed in Patient W.N., who was unresponsive to both Su-4885 and dexamethasone, in order to determine whether the pituitary of such a patient could respond to other stimuli known to increase ACTH under normal circumstances. Specifically, the responses to corticotropin-releasing factor (CRF, "fraction D" of Guillemin) and bacterial pyrogen were compared to the effect of ACTH administered intravenously. These studies were conducted on four successive days on a metabolic ward. Plasma 17-OH-CS values are shown in Figure 3, and the concomitant 12-hour urinary 17-KGS and 17-KS values are listed in Table 2.

On the first day, 250 ml. of saline diluent for CRF was infused over a

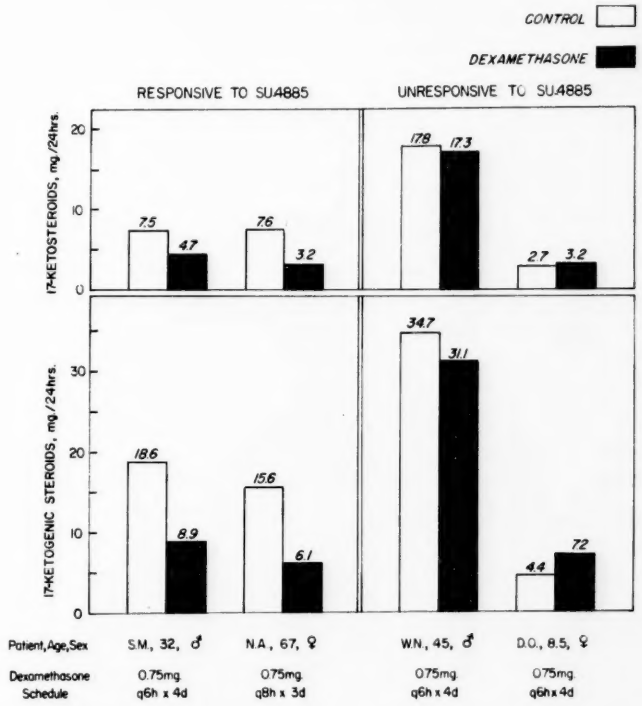

FIG. 2. Dexamethasone suppression tests in 2 patients responsive to Su-4885 and in 2 patients unresponsive to Su-4885. The white columns represent the control steroid excretion on the day preceding administration of dexamethasone. The black columns represent steroid excretion on the fourth day of dexamethasone administration.

TABLE 2. SPECIAL STUDIES OF PITUITARY RESPONSIVENESS IN PATIENT W.N.—URINARY EXCRETION OF 17-KGS AND 17-KS*

Collection period	Procedure	17-KGS (mg./24 hrs.)	17-KS (mg./24 hrs.)
April 6, 1 p.m. to April 7, 1 a.m.	Saline diluent i.v. (2:30–6:30 p.m.)	13.7	10.4
April 7, 1 a.m. to April 7, 1 p.m.		9.4	12.0
April 7, 1 p.m. to April 8, 1 a.m.	CRF (fraction D), 20 mg. i.v. in 250 ml. saline (2:30–6:30 p.m.)	10.5	9.0
April 8, 1 a.m. to April 8, 1 p.m.		9.0	11.6
April 8, 1 p.m. to April 9, 1 a.m.	ACTH, 25 units i.v. (2:30–6:30 p.m.)	24.7	17.3
April 9, 1 a.m. to April 9, 1 p.m.		18.1	23.2
April 9, 1 p.m. to April 10, 1 a.m.	Piromen injected i.v., 3:15 p.m.	32.1	40.0
April 10, 1 a.m. to April 10, 1 p.m.		19.8	26.2

* Successive 12-hour determinations of 17-KGS and 17-KS in a patient who was unresponsive both to Su-4885 and dexamethasone. Corresponding levels of plasma unconjugated 17-OH-CS indicated in Figure 3.

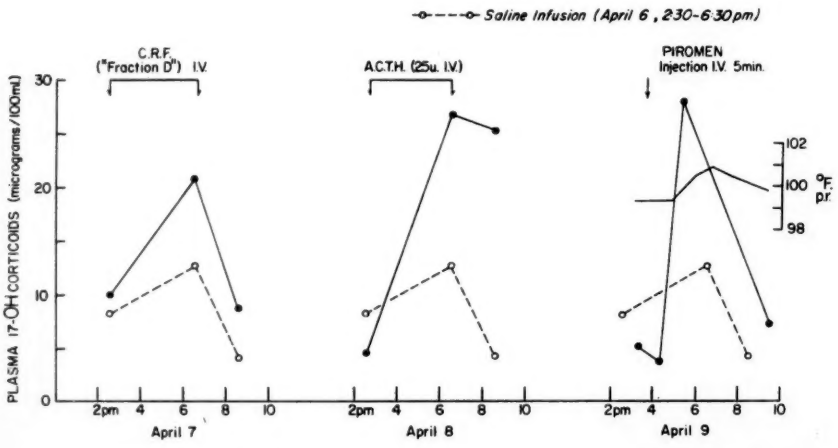

FIG. 3. Plasma 17-OH-CS responses to intravenous infusion of saline, CRF (Guillemin), ACTH, and bacterial lipopolysaccharide (Piromen) in Patient W. N. Observations during the infusion of saline on April 6 were considered to be appropriate control values, and are indicated by the broken line. For steroid excretion values, see Table 2.

period of four hours. The plasma 17-OH-CS levels were employed as a control for subsequent studies. In Figure 3 the plasma 17-OH-CS values on this day are connected by a broken line.

On the second day of this study, 20 mg. of CRF (standard "fraction D," batch No. 300-6, kindly supplied by Dr. Roger Guillemin) was dissolved in 10 ml. of 0.6 per cent acetic acid and infused in 250 ml. of saline over a four-hour period. Previous studies with this material in human beings have been reported (24). CRF induced a rise in the plasma 17-OH-CS level above that induced by the control infusion. This response, however, was apparently not sufficiently prolonged to be reflected in the 12-hour urinary steroid values. No unusual subjective phenomena or changes in vital signs accompanied this infusion.

On the third day, a standard intravenous infusion of ACTH elicited the expected rise in the level of plasma 17-OH-CS and urinary 17-KGS and 17-KS. On the fourth day, a bacterial lipopolysaccharide pyrogen (Piromen), first employed by Bliss and co-workers (25) as a standard febrile stimulus of ACTH release, was injected intravenously (0.5 μg. per Kg.) over a period of five minutes. A prompt adrenal response was reflected in both urinary and plasma values. Other than the expected transient fever and chills, no untoward side-effects accompanied this procedure.

DISCUSSION

Three patients suffering from CNS disease but with apparently intact pituitaries and normal adrenal function were found to exhibit a derangement of normal pituitary-adrenal interrelationships. These patients failed to demonstrate a rise in ACTH secretion in response to a fall in circulating cortisol, as judged by their failure to respond to Su-4885 with the expected increase in urinary 17-KGS. Conversely, 2 of these patients failed to demonstrate the anticipated suppression of endogenous ACTH secretion by elevated levels of glucocorticoids, as inferred from the inability of dexamethasone to induce a fall in urinary steroids. The third patient was not tested with dexamethasone. These findings suggested the possibility that in these patients there was a disturbance of the CNS centers responsible for adjusting the secretion of ACTH by the pituitary to varying levels of circulating glucocorticoids. The inadequate localization of lesions in these patients frustrated any attempt at a more precise anatomic definition of these centers.

Two of the patients (D.O. and W.N.) unresponsive to Su-4885 were being treated with the anticonvulsant diphenylhydantoin, at the time of these studies. This drug has been reported to interfere with adrenocortical function (26, 27), with the response of plasma steroids to exogenous ACTH

(28, 29), and with the release of pituitary ACTH (30). Furthermore, Gold, Kent and Forsham (31) have shown that certain psychotropic drugs can modify the response to Su-4885. Thus the possibility must be kept in mind that the results obtained in these patients were due to diphenylhydantoin rather than to the primary cerebral disease. This explanation does not appear likely, however, because one of the patients (P.vT.) unresponsive to Su-4885 had never received this drug, and 4 patients (J.K., K.Mc., C.F. and T.S.) who were treated with it did respond to Su-4885. Also those patients who were taking diphenylhydantoin and were unresponsive to Su-4885 had a normal response to exogenous ACTH and a normal baseline excretion of 17-KGS.

Hökfelt and Luft (32) recently studied adrenocortical function in 10 patients with suprasellar tumors. Eight of these patients, however, had demonstrable intrasellar disease. Three of the 4 patients tested with Su-4885 exhibited responses which were interpreted as normal. The fourth patient had a low baseline urinary steroid excretion, and the failure to respond to Su-4885 may therefore have reflected adrenal atrophy.

Previous studies in animals have attempted to determine whether steroids act directly on the pituitary or on some CNS site in controlling the secretion of ACTH. Evidence for a direct pituitary action are the observations indicating that a normal reciprocal pituitary-adrenal relationship can exist in the stalk-sectioned rabbit (33) and in hypophysectomized rats bearing pituitary homografts under the renal capsule (34). However, the lack of direct anatomic continuity between hypothalamus and pituitary does not preclude a systemic distribution of neurohumors. Rose and Nelson (35) showed that hydrocortisone infused directly into the pituitary fossa of rats leads directly to pituitary atrophy; retrograde flow of hydrocortisone into the central nervous system could, however, not be entirely discounted. Ganong and Hume (36) reported that larger amounts of cortisol could induce adrenal atrophy in dogs with lesions in the median eminence. On the other hand the same authors (37) showed that unilateral adrenalectomy failed to induce compensatory adrenal hypertrophy in dogs with hypothalamic lesions. Essentially similar results in the rat have been reported by McCann and co-workers (38–40). The latter data are more consistent with the concept of a cerebral control mechanism. Also favoring this thesis is the claim by Schapiro, Marmorston and Sobel (41) that a neurosecretory humor in hypophysectomized rats can be blocked by desoxycortisone acetate. Since these authors employed the fall in circulating eosinophils as an index of adrenocortical activity, the conclusions drawn must await confirmation by more specific tests of adrenocortical function.

Of interest in the present study is the observation that Patient W.N. could respond with a prompt rise in plasma and urinary corticoids to the

intravenous injection of bacterial pyrogen, despite the fact that he was unresponsive to both Su-4885 and dexamethasone. This is in accord with the previously stated thesis (42) that different mechanisms are involved in the homeostatic control of the level of circulating corticoids under baseline conditions, and in situations where there is increased adrenocortical secretion in response to stressful stimuli. Supporting the existence of this dichotomy is the observation of Van Wyk, Dugger and Newsome (43) that certain patients with pituitary stalk sections failed to respond to Su-4885 but retained their ability to react to pyrogen and insulin hypoglycemia with a rise in the level of plasma corticoids. Also, Gold, Kent and Forsham (31) found that patients treated with certain psychotropic drugs had normal adrenal responses to surgery in the presence of impaired responses to the Su-4885 test.

Since Patient W.N. was capable of increasing ACTH secretion in response to a stressful stimulus and since Patients D.O. and P.vT. manifested no clinical evidence of limited adrencortical-pituitary "reserve," the question arises as to what mechanism is brought into play to shut off the secretion of ACTH after the cessation of the stressful stimulus. Presumably some mechanism other than the normal inhibitory influence of elevated levels of circulating cortisol is involved. It may be of interest in this connection that Patient W.N. occasionally excreted elevated quantities of urinary 17-KGS. In the dexamethasone suppression test, the baseline 24-hour excretion of 17-KGS was 34.7 mg., a moderately elevated value. However, there was no clinical suggestion of adrenocortical overactivity. It may be that the postulated alternative shut-off device is not as effective as the normally operative mechanism and thus could account for a wider degree of variation in the adrenal secretion of cortisol.

The mechanism that maintains the normal diurnal variation in plasma 17-OH-CS apparently is not dependent on the normal functioning of those CNS structures postulated to control the reciprocal adrenal-pituitary relationship. Two of the patients (P.vT. and W.N.) who were unresponsive to Su-4885 exhibited the normal diurnal variation in plasma 17-OH-CS. In contrast, Hökfelt and Luft (32) observed a lack of diurnal 17-OH-CS variation in 4 of 8 patients. However, 3 of these 4 patients had intrasellar tumors. Thus it is difficult to ascertain whether the intrinsic pituitary damage or the suprasellar extension accounted for the observed derangement.

Of incidental interest in the present study is the fact that 2 patients normally responsive to Su-4885 had diabetes insipidus. Patient S.M. was maintained with Pitressin, but Patient A.Th. received no replacement therapy. These observations add additional weight to the argument that vasopressin is not the neurohumor that stimulates the ACTH-producing cells of the anterior hypophysis.

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THE EFFECT OF IODIDES ON HASHIMOTO'S THYROIDITIS*

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ABSTRACT

Eighteen patients with Hashimoto's thyroiditis and 4 with a solitary hyperfunctioning thyroidal nodule were given I^{131} before and after administration of 2000 μg. of stable iodide as potassium iodide. The difference between the 24-hour thyroidal uptakes of the pre-potassium iodide and post-potassium iodide tracers was reduced to a percentage in the manner described by Feinberg and associates. The percentage inhibition of thyroidal I^{131} uptake in all the cases studied was striking, and differed appreciably from the values for normal persons. It simulated the findings in Graves' disease and iodide goiter. The administration of TSH to normal subjects did not consistently alter the pattern of response to the "iodide inhibition test." From these preliminary studies it is postulated that iodides enhance a pre-existing intrathyroidal defect that is conceivably related to the organification of iodide.

SINCE Plummer's employment of iodides in the therapy of Graves' disease (1), their clinical usefulness for this disorder has been universally documented, but knowledge of their mode of action remains incomplete. It has been shown by Wolff and Chaikoff (2) in the rat and by Stanley (3) and Childs and co-workers (4) in man that elevated concentrations of serum iodide are associated with inhibition of organic binding of iodine in the thyroid gland. Stanley's studies demonstrated the increased susceptibility of the thyroid of patients with Graves' disease to inhibition by iodide. These observations were extended by Feinberg and associates (5), who devised a test of diagnostic usefulness in which the uptake of I^{131} by the thyroid is measured before and after the administration of variable doses of carrier I^{127}. A dose of 2000 μg. of iodide administered with the second tracer induced a significantly greater diminution in the thyroidal uptake of I^{131} in patients with Graves' disease than in euthyroid controls. Utilizing a comparable technic, Paris and associates (6) found that pa-

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tients with iodide goiters reacted to the "iodide inhibition test" as did patients with Graves' disease. The present communication constitutes a preliminary report on the effect of iodides on the thyroidal uptake of I^{131} in patients with Hashimoto's thyroiditis and functioning thyroid adenomas.

SUBJECTS AND METHODS

The patients included in the study were those in whom the diagnosis of Hashimoto's thyroiditis had been made since the spring of 1960. Cases of Hashimoto's thyroiditis not proven by biopsy material from the thyroid gland were excluded. The status of thyroidal function in these patients was ascertained on clinical grounds and from determination of the basal metabolic rate (BMR) and serum protein-bound iodine (PBI) level. Whenever possible, the concentration of butanol-extractable iodine (BEI) was also determined. The patients with solitary hyperfunctioning nodules were selected only after palpation of the thyroid gland excluded multiple nodularities and the gammagram indicated an exclusive or predominant uptake of I^{131} over the nodule; the presence of parenchymatous hypertrophy was ruled out by needle biopsy or study of material obtained at operation. Euthyroid controls were selected from personnel and patients of the Mayo Clinic. The criteria for acceptability were willingness to co-operate and the presence of a euthyroid state and a normal thyroid gland on palpation.

The iodide inhibition test was performed in accordance with the protocol outlined by Feinberg and associates (5). Their data documented the fact that 2 mg. of potassium iodide (KI) raises the concentration of serum iodide to the neighborhood of 6 μg. per 100 ml., irrespective of the patient's thyroidal function.

Thyroid-stimulating hormone (TSH) (Armour, lot no. V4508) was administered to normal persons approximately two weeks after a preliminary iodide inhibition test. The TSH was given subcutaneously in a 5-unit dose on two consecutive days. On the day after the last administration of TSH, the iodide inhibition test was repeated. (Serial I^{131} tracer studies performed daily during a "scouting" experiment showed an appreciable elevation in thyroidal I^{131} uptake on the fourth and fifth days after the last dose of TSH.)

RESULTS

The pertinent data in the cases of Hashimoto's thyroiditis are summarized in Table 1. The "inhibition" of thyroidal uptake of I^{131} induced by 2000 μg. of iodide (as KI) was striking. It should be noted that the degree of inhibition was pronounced irrespective of the initial uptake of I^{131} by the thyroid gland.

Table 2 shows the results of the "iodide inhibition test" in 10 normal subjects. The extreme variations are obvious, but it should be noted that the "inhibition" did not exceed 54 per cent; the mean value for the percentile inhibition was 35 ± 16.7 (1 S.D.). Comparable studies in 11 euthyroid controls by Feinberg (7) revealed a mean percentile inhibition of 40 ± 19.3 (1. S.D.).

Table 3 shows the pertinent data and the results of the iodide suppression test in 4 patients with hyperfunctioning thyroidal nodules. The degree of suppression was pronounced and uniform.

TABLE 1. IODIDE INHIBITION TEST IN HASHIMOTO'S THYROIDITIS

Patient		Clinical data	PBI (μg./100 ml.)	BEI (μg./100 ml.)	BMR (%)	24-Hour thyroidal I^{131} uptake		Percentile fall*
No.	Sex & age (yrs.)					Before KI (% of dose)	After KI (% of dose)	
1	F 40	Goiter and myxedema appeared after iodide therapy was begun. Iodides stopped 10 days before tests.	6.2	4.5	−26	75	12	84
2	F 44	Goiter first noted during recent pregnancy.	3.0	2.0	− 5	31	4	87
3	F 45	Goiter unknown before admission.	5.4	—	+ 1	26	2	92
4	M 13	Goiter present 2 yrs. and treated irregularly with iodides.	7.0	—	—	26	12	54
5	F 43	Goiter unknown before admission. Mild myxedema present.	1.7	0.7	−19	8	2	75
6	F 48	Subtotal thyroidectomy for "toxic goiter" 23 yrs. previously. Myxedema and recurrent goiter appeared 3 yrs. before admission.	2.0	1.6	−25	15	1	93
7	F 52	Goiter unknown before admission. Myxedema present.	1.8	0.8	−27	8	2	75
8	F 60	Goiter unknown before admission.	4.0	—	—	22	4	82
9	F 33	Goiter present 7 yrs. Myxedematous.	1.4	—	−13	11	2	82
10	F 20	Goiter present 5 mos. Euthyroid.	4.1	2.9	− 7	23	3	87
11	F 38	Goiter found in pre-employment examination. Euthyroid.	—	—	—	17	4	76
12	F 22	Goiter present 2 mos. Euthyroid.	3.3	2.6	0	59	6	90
13	F 31	Goiter present 1 yr. Euthyroid.	4.3	3.5	− 4	50	1	98
14	F 62	Myxedema present 10 yrs. Unaware of diffuse, firm, 25-gm. goiter.	1.2	—	−25	18	1	93
15	F 48	Goiter present 2 yrs. Euthyroid. Brother and 3 daughters had Graves' disease. Mother had large goiter.	4.8	—	−11	17	7	59
16	F 44	Goiter and myxedema discovered while vague neck discomfort was being evaluated.	1.7	0.7	−19	11	1	99
17	F 47	Goiter 20 yrs.; recent enlargement in conjunction with iodide administration. Euthyroid.	5.5	2.5	0	50	9	88
18	F 58	Goiter of long duration. Mild hypothyroidism. Father and sister goitrous.	2.8	—	− 7	59	5	90

* Percentile fall $= \frac{\text{Initial 24-hour uptake minus final 24-hour uptake}}{\text{Initial 24-hour uptake}} \times 100$. Mean percentile fall $= 84 \pm 12.5$ (1 S.D.).

COMMENT

The inhibition by iodides of the thyroidal uptake of I^{131} in Hashimoto's thyroiditis was to us a rather fortuitous finding. The first patient reported in this series was seen because of a goiter and myxedema. She had been given Lugol's solution a few months earlier as therapy for nervousness, and soon thereafter noted pronounced enlargement of the thyroid gland and the onset of sluggishness, puffiness of the face, and intolerance to cold. Our initial diagnosis was iodide goiter with mild myxedema. However, a needle biopsy of the thyroid gland revealed the presence of classic Hashimoto's thyroiditis. It was then conjectured that the fundamental derangement of

TABLE 2. EFFECT OF 2000 μG. OF KI ON THYROIDAL I^{131} UPTAKE IN EUTHYROID CONTROLS

Subject	24-Hour thyroidal I^{131} uptake		
	Before KI (% of dose)	After KI (% of dose)	Percentile fall*
1	20	9	54
2	38	24	37
3	41	22	46
4	23	11	52
5	25	12	52
6	23	12	47
7	13	10	23
8	19	17	10
9	25	23	8
10	29	23	21

* Percentile fall $= \frac{\text{Initial 24-hour uptake minus final 24-hour uptake}}{\text{Initial 24-hour uptake}}$. Mean percentile fall $= 35 \pm 16.7$ (1 S.D.).

thyroidal function that rendered patients with Graves' disease and iodide goiter so readily susceptible to the inhibitory effects of iodine was perhaps shared by other disease entities such as Hashimoto's thyroiditis. Dowling and Becker (8) recently reported the case of a 67-year-old woman with myxedema and goiter which were presumably secondary to the intake of hydriodic acid. Partial recovery occurred when iodides were discontinued, and defective organification of iodide was then demonstrated. The eventual spontaneous occurrence of myxedema attended by the disappearance of

TABLE 3. IODIDE INHIBITION TEST IN FUNCTIONING THYROID ADENOMATOUS NODULES

Patient		Clinical data	PBI (μg./100 ml.)	BEI (μg./100 ml.)	BMR (%)	24-Hour thyroidal I^{131} uptake		
No.	Sex & age (yrs.)					Before KI (% of dose)	After KI (% of dose)	Percentile fall*
19	F 50	Single nodule 4 cm. in diameter in left lobe of thyroid. Gammagraphic evidence of predominant collection of I^{131} by nodule. Macrofollicular adenoma at operation.	5.3	—	—	41	8	81
20	F 69	Single nodule 2.5 cm. in diameter in right lobe of thyroid. Gammagraphic evidence of exclusive collection of I^{131} by nodule. Mild hyperthyroidism. Macrofollicular adenoma at biopsy.	8.3	—	+13	37	13	65
21	F 70	Previously unaware of goiter. Single nodule 4 cm. in diameter in left lobe of thyroid. Gammagraphic evidence of exclusive collection of I^{131} by nodule. Mild hyperthyroidism. Macrofollicular adenoma at operation.	7.6	—	+36	46	6	87
22	M 63	Single nodule 6 cm. in diameter in right lobe of thyroid. Gammagraphic evidence of exclusive collection of I^{131} by nodule. Mild hyperthyroidism of 1 year's duration. Single, cystic macrofollicular adenoma at operation.	7.8	7.1	+22	43	9	79

* Percentile fall $= \frac{\text{Initial 24-hour uptake minus final 24-hour uptake}}{\text{Initial 24-hour uptake}} \times 100$.

the goiter suggested to these workers that their patient might have suffered from underlying Hashimoto's thyroiditis. Dowling and Becker (8) proposed the use of iodides to bring out defects in organic iodination. Feinberg and co-workers (5) had studied 2 cases of Hashimoto's thyroiditis, with less conclusive results. One of their patients showed a meager inhibition comparable to that seen in persons with normal thyroids, but the amount of carrier iodide used was only 750 μg. In the second patient a carrier dose of 1500 μg. of KI was used, and the suppression approximated that seen in Graves' disease.

The mechanism of the inhibition of the thyroidal uptake of I^{131} by iodides remains unexplained. In a detailed study of the metabolism of iodide, Feinberg and co-workers (5) found a pronounced increase in the thyroidal accumulation of inorganic iodide when a carrier dose of 2000 μg. was administered to euthyroid persons. This increase was not attended by significant changes in the serum protein-bound radioiodine (PBI^{131}) level at twenty-four hours. In some of the patients with Graves' disease the initial accumulation of inorganic iodide in the thyroid gland was considerably higher than normal, but when they were given the KI load, the thyroidal accumulation of iodide increased proportionately much less than in normal subjects, and in one instance it actually decreased. The values for serum PBI^{131} and butanol-extractable radioiodine (BEI^{131}) after the KI load were not uniform, but in some cases of Graves' disease the output of PBI^{131} from the thyroid was stopped entirely by administration of KI.

Additional experiments were designed to ascertain whether iodides truly induced a supranormal inhibition of thyroidal I^{131} uptake in the disease states under consideration, or whether this superinhibition simply reflected a more efficient mechanism for intrathyroidal iodide concentration in Graves' disease, iodide goiter, and Hashimoto's thyroiditis than in the normal situation. It was postulated that increasing the trapping ability of the normal thyroid gland with exogenous TSH would induce a greater concentration of iodide intrathyroidally, thus multiplying the carrier dose potential in terms of inhibition.

Our observations neither support nor refute this hypothesis (Table 4). TSH induced thyroidal overactivity in all subjects, as evidenced by tachycardia, stimulation, hyperhydrosis, and varying degrees of thyroidal enlargement, yet the pattern of iodide inhibition in 3 of 5 subjects was similar before and after TSH. Subjects 4 and 5 responded differently. More observations with the iodide inhibition test before and after administration of TSH in normal persons will be needed to ascertain whether the magnitude of the response to TSH in terms of increased thyroidal uptake of I^{131} and the degree of inhibition induced by iodides thereafter are directly related.

That patients with hyperfunctioning solitary thyroid nodules should

TABLE 4. EFFECT OF EXOGENOUS TSH ON IODIDE INHIBITION TEST IN NONGOITROUS, EUTHYROID SUBJECTS

Subject	24-Hour thyroidal uptake of I^{131}		
	Before KI (% of dose)	After KI (% of dose)	Percentile fall*
1 Before TSH	38	24	37
After TSH	49	37	37
2 Before TSH	25	12	52
After TSH	36	20	50
3 Before TSH	23	11	52
After TSH	41	15	63
4 Before TSH	19	17	10
After TSH	67	25	63
5 Before TSH	29	23	21
After TSH	68	17	75

$$* \text{ Percentile fall} = \frac{\text{Initial 24-hour uptake minus final 24-hour uptake}}{\text{Initial 24-hour uptake}} \times 100.$$

have exhibited as pronounced an inhibition in I^{131} thyroidal uptake after KI as did patients with Graves' disease, iodide goiter, or Hashimoto's thyroiditis militates against any exclusive role of excess TSH in the mediation of iodide inhibition. It does not negate the possibility that a relative or absolute increase in the capacity to concentrate iodide intrathyroidally separates the group of patients under consideration from normal persons. Such a mechanism would be secondary to pre-existing abnormalities in thyroidal function, such as the faulty organification of iodide demonstrated by Morgans and Trotter (9) in Hashimoto's thyroiditis.

Autoradiographic and chromatographic studies are currently being performed on tissue obtained at operation and on needle biopsy specimens from patients with Hashimoto's thyroiditis and from those with hyperfunctioning adenoma. The results of these observations will be the object of a more detailed report.

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DISSOCIATION OF RESPONSE TO TRIIODOTHYROACETIC ACID IN MYXEDEMA: COMPARISON WITH RESPONSE TO THYROID SUBSTANCE*

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ABSTRACT

In order to confirm the previously reported dissociation of the physiologic effects produced by the acetic acid derivatives of thyroxine and of triiodothyronine in patients with myxedema, additional studies of the action of triiodothyroacetic acid (Triac) were made over a prolonged period of time, concentrating on the response of the BMR and the serum cholesterol level. Five patients with myxedema received Triac and 6 patients received thyroid substance. Each compound was administered in increasing dosage and the effect of each dosage was observed for a period of two to three months. The daily dose of Triac varied from an initial 0.5 mg. to a maximum of 4.0 mg. The daily dose of thyroid varied from an initial 15.0 mg. to a maximum of 150.0 mg. in 4 patients; the other 2 patients received only 15.0 mg. daily. In general, dosages of Triac up to 2.0 mg. daily failed to produce a rise in BMR, whereas the concentration of serum cholesterol dropped 30 to 40 per cent from the pretreatment level. With thyroid, the changes in BMR and serum cholesterol were proportional as the dosage of thyroid was increased, with 1 exception; a patient tested only at the 15.0-mg. dose level also showed a dissociation between the BMR and the concentration of serum cholesterol. The dissociation between the BMR response and the cholesterol response also holds for the propionic acid derivatives, but not for the formic acid derivatives. As a result of its mode of action, Triac may prove to be superior to thyroid in the management of myxedema complicated by angina.

FOLLOWING the report of Thibault and Pitt-Rivers (1) that the action of triiodothyroacetic acid (Triac) and of tetraiodothyroacetic acid (Tetrac) in animals and in kidney slices is rapid in onset and short in duration, Lerman and Pitt-Rivers (2) found the action of these compounds in human myxedema to be as follows: 1) single doses cause an appreciable rise in the basal metabolic rate (BMR) during the first few hours, a rise which tends to subside at the end of twenty-four hours; 2) small daily doses of Triac (less than 2.0 mg.) induce a distinct drop in the concentration of serum cholesterol, an increase in the urinary excretion of creatine, a return of the electrocardiographic pattern to normal, an appreciable weight loss,

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an improvement in the myxedematous appearance, but no alteration in the BMR. We had never observed such a dissociation of response in the course of assaying numerous analogues in patients with myxedema.

In 1956 Trotter (3) reported that the relative effects of Triac and triiodothyronine on the blood cholesterol level and on the BMR appeared to be similar in 2 patients with myxedema; however, in another case, Triac in a dosage of 4.0 mg. daily for three weeks appeared to have a proportionately greater effect on the cholesterol level relative to the BMR than did thyroxine. In one of the 2 cases mentioned, the initial daily dose of 6.0 mg. of Triac produced a definite rise in BMR as well as a drop in the concentration of blood cholesterol, but when the dosage was reduced to 2.0 mg. daily for a period of about three weeks, the BMR returned to pretreatment levels whereas the blood cholesterol concentration rose only slightly from its lowest point. De Graeff *et al.* (4) were able to confirm the dissociation between the basal metabolic rate and the serum cholesterol level in a patient with myxedema receiving 1.0 mg. of Triac per day intravenously for twelve days. Boyd and Oliver in 1957 (5) administered 0.5 mg. of Triac daily for seventeen days to a hypothyroid patient and found that the serum cholesterol level dropped from 500 mg. per 100 ml. to 325 mg. per 100 ml. without a rise in BMR or a change in weight. On the other hand, 2 patients with myxedema showed a marked rise in BMR and a corresponding weight loss, as well as a drop in the concentration of blood cholesterol while receiving 4.0 mg. daily for six days. Goolden (6) in 1956 had reported that a patient with myxedema had responded to Tetrac (in a dosage beginning with 3.0 mg. and increased to 6.0 mg. daily for two weeks), both the BMR and the serum cholesterol level returning to normal values. Wiswell and Asper (7), studying 3 patients with myxedema, found that the effects of Triac and Tetrac on oxygen consumption and on the metabolism of cholesterol, nitrogen, phosphorus and creatine resembled closely those of triiodothyronine. Recently Hill *et al.* (8) found that, in patients with myxedema, small daily doses of Triac (1 to 3 mg.) and of Tetrac (2 to 4 mg.) produced a distinct reduction in the serum cholesterol level without a rise in BMR. However, they demonstrated a similar dissociation in the effects of triiodothyronine.

The possibility that the physiologic effects of the acetic acid derivatives of thyroxine and of triiodothyronine may be dissociated led us to study the action of Triac more extensively over a prolonged period of time, concentrating primarily on the BMR and the serum cholesterol level. The results were compared with those of small and increasing doses of whole thyroid on patients with hypothyroidism. Other aspects of the response of myxedema to acetic acid derivatives have been described in a previous report (2).

MATERIAL AND METHODS

Five patients with primary myxedema received Triac, and 6 received thyroid substance.[1] The initial dosage of Triac was 0.5 mg. daily, except in 1 case in which it was 1.0 mg. daily. The daily dose was increased by increments of 0.5 to 1.0 mg. every two to three months. One patient (Fig. 1) also received Tetrac for a short period of time. The initial dosage of thyroid in 2 patients was 15.0 mg. daily, increased by an increment of 15.0 mg. every two to three months. In 2 patients the initial dosage was 30.0 mg. daily, increased by an increment of 30.0 mg. every two to three months. Two additional patients were tested only at the 15.0-mg. dose level.

At each visit (every one to three weeks) the clinical changes were evaluated, and a BMR and serum cholesterol determination were obtained.

RESULTS

The results of the administration of Triac are depicted in Figures 1 and 2. Figure 1 shows the response of a myxedematous patient to Triac and

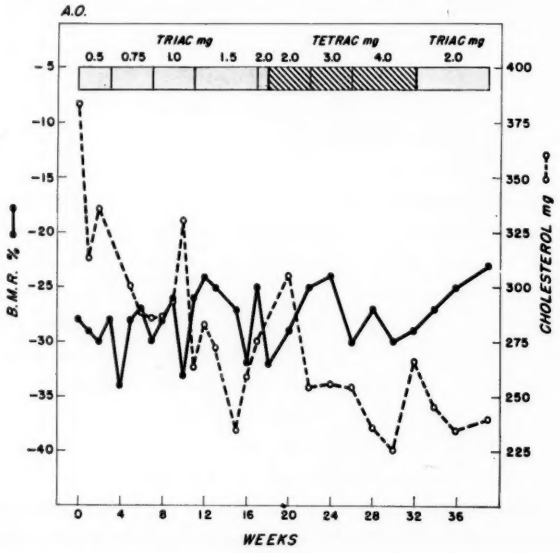


FIG. 1. The response of the BMR and serum cholesterol level to Triac and Tetrac in a patient with myxedema.

Tetrac. With a dosage of 0.5–1.5 mg. of Triac daily for sixteen weeks there was little or no change in the BMR whereas the concentration of serum cholesterol dropped about 30 per cent from the pretreatment level. Administration of Tetrac for fourteen weeks in a dosage of 2.0–4.0 mg. daily failed to change the BMR and produced a slight further drop in serum cholesterol concentration. Finally, when Triac was given in dosage of 2.0

[1] Thyroglobulin. The commercial preparation used was Proloid®.

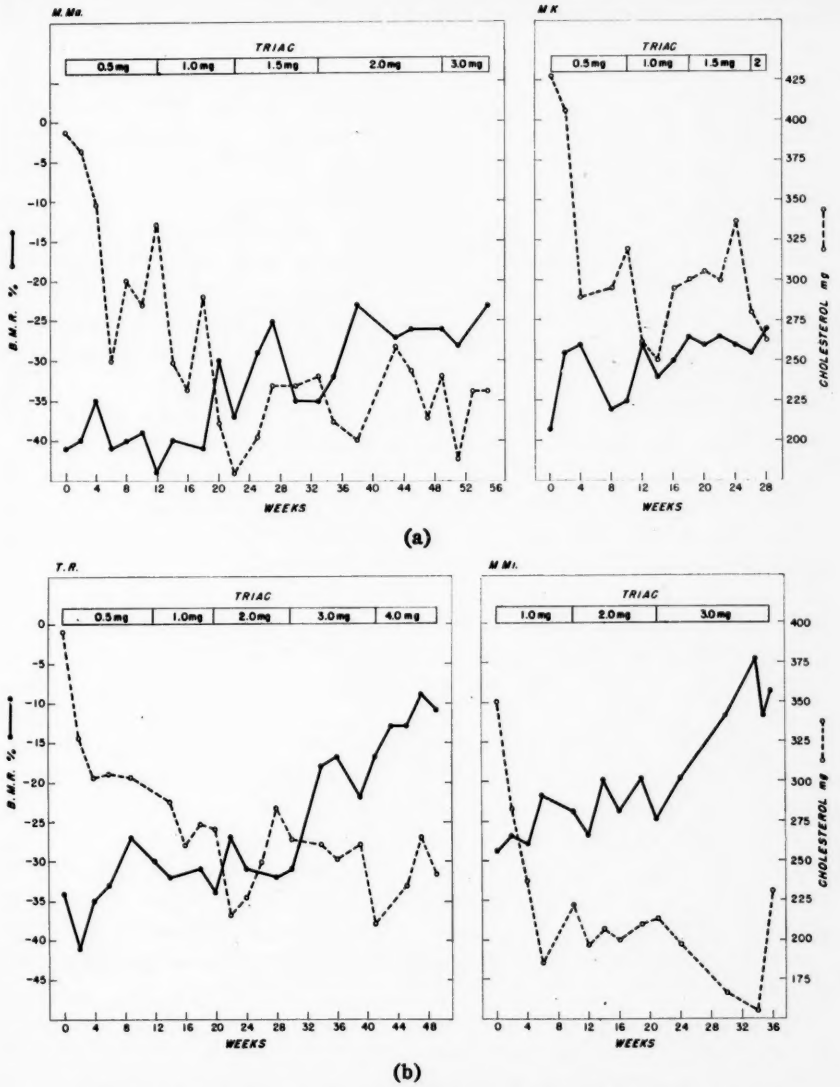


FIG. 2 (**a** and **b**). The response of the BMR and serum cholesterol level to increasing doses of Triac in 4 patients with myxedema.

mg. daily for seven weeks, the BMR rose a few points.

Figure 2 (a and b) shows the response of 4 patients with myxedema to Triac. In Patient M.Ma there was a slight but unsustained rise in the BMR with 1.5 mg. daily, from a level of −41 to a level of −35 per cent. During

this period the serum cholesterol concentration dropped about 40 per cent from its pretreatment value. With 2.0 mg. of Triac daily there was a distinct rise in BMR.

Patient M.K. (Fig. 2a) showed no metabolic response with a dosage of 0.5 mg. of Triac daily, while the serum cholesterol concentration dropped about 30 per cent from the pretreatment level. With dosages of 1.0 mg. and 1.5 mg. daily there was a slight rise in BMR (about 10 points).

Patient T.R. (Fig. 2b) showed an equivocal rise in BMR with dosages of Triac up to 2.0 mg. daily for thirty weeks, during which time the serum cholesterol level dropped over 30 per cent from the pretreatment value, and for a time even lower than that. With dosages of 3.0 mg. and 4.0 mg. daily there was a progressive rise in BMR without further change in the serum cholesterol level.

Patient M.Mi. (Fig. 2b) exhibited an equivocal metabolic response (5–6 points) with dosages of 1.0 and 2.0 mg. of Triac over a period of twenty-one weeks, during which time the serum cholesterol level dropped about 40 per cent from pretreatment value. With a dosage of 3.0 mg. of Triac daily there was a progressive rise in the BMR and a further drop in cholesterol concentration, although the latter was not sustained.

In general, in patients with myxedema, dosages of Triac up to 2.0 mg. daily failed to produce a rise in BMR, whereas the serum cholesterol concentration dropped 30–40 per cent from its pretreatment level.

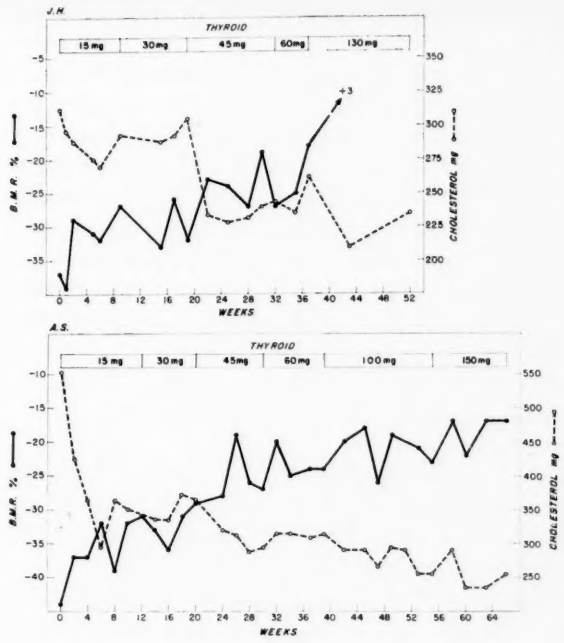
The response of patients with myxedema to thyroid hormone is shown in Figure 3, a and b.

Patient J.H. (Fig. 3a) showed a steady, slow rise in BMR and a corresponding slow drop in serum cholesterol concentration as the dosage of thyroid was increased from 15.0 mg. to 130 mg. daily.

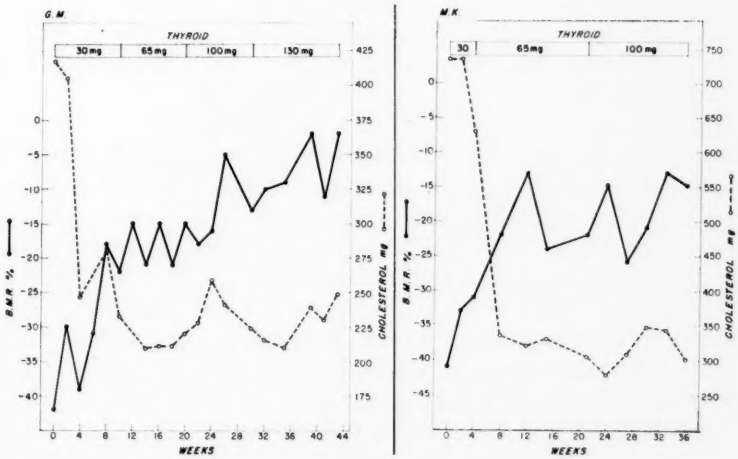
Patient A.S. (Fig. 3a) showed the same phenomenon. Although the initial 15-mg. dosage produced a considerable drop in the serum cholesterol level (about 35 per cent), there was also a fairly brisk rise in the BMR of about 15 points (or a relative change of 25 per cent). With subsequent dosages, the changes in both BMR and serum cholesterol were slight but progressive.

Patient G.M. (Fig. 3b) manifested a progressive rise in BMR and a progressive fall in serum cholesterol concentration as the dosage of thyroid was increased from 30.0 mg. to 100.0 mg. daily. This patient demonstrated the occasionally observed peculiar phenomenon of a paradoxical rise in the serum cholesterol level as the dosage of thyroid is increased. In this case the cholesterol concentration tended to rise during treatment with 130.0 mg. daily, instead of dropping further.

Patient M.K. (Fig. 3b) also showed a proportionality between the drop in serum cholesteral concentration and the rise in BMR. Thus 4 patients



(a)



(b)

FIG. 3 (**a** and **b**). The response of the BMR and serum cholesterol level to increasing doses of thyroid hormone in 4 patients with myxedema.

demonstrated proportionality between the changes in BMR and serum cholesterol concentration as the dosage of thyroid was increased over a period of months. However, 2 additional patients tested only at the 15.0-mg. dosage level of thyroid, behaved differently. In one, in whom it was difficult to obtain a satisfactory reading, the BMR ranged between −2 and −7 per cent both before and after treatment, whereas the serum cholesterol level dropped from 360 mg. to 234 mg. per 100 ml. over a period of two months. In the second patient, a BMR of −40 per cent remained at this level for six weeks, whereas the serum cholesterol level dropped from 362 mg. to 247 mg. per 100 ml. It seems, therefore, that at this dosage level at least, one patient showed the same dissociation between BMR and serum cholesterol responses that was observed in patients treated with the acetic acid derivatives.

DISCUSSION

The results reported previously by Lerman and Pitt-Rivers (2) on the action of Triac and Tetrac in myxedema suggested the possibility that changes in the chemical structure of the thyroxine molecule may affect the various physiologic actions of thyroxine differently, *i.e.*, dissociate them. This concept is supported by the findings of various investigators that most analogues have different potencies, dependent upon the assay procedures used, such as tadpole metamorphosis, inhibition of radioactive iodine uptake, or antigoitrogenesis. The results reported here tend to confirm the previous ones that dosages of Triac under 2.0 mg. daily cause a marked reduction in the serum cholesterol level without a corresponding change in the basal metabolic rate.

The nature of the response can be better demonstrated by comparing the percentage changes in each parameter in the 5 patients receiving increasing doses of Triac with those in the 4 patients receiving increasing doses of thyroid. The daily dose of Triac varied from an initial 0.5 mg. to a maximum of 4.0 mg. With the use of data previously reported by Lerman and Pitt-Rivers (2) and by Rawson *et al.* (9), it can be calculated that 0.5 mg., 1.0 mg. and 1.5 mg. of Triac are equivalent to 45 mg., 90 mg. and 130 mg. respectively of thyroid substance. As shown in Table 1 the percentage change in BMR in response to the administration of Triac was negligible when the daily dose was less than 2.0 mg., and was only slight at the 2.0-mg. level, whereas the major portion of the cholesterol reduction had taken place by the time this dosage was reached. Administration of thyroid (except in 1 patient tested only with 15 mg.) produced distinct and almost parallel changes in the BMR and in the serum cholesterol level with increasing dosage.

The plotted data representing the change in BMR with increasing doses

TABLE 1. AVERAGE PERCENTAGE CHANGE IN BMR AND SERUM CHOLESTEROL LEVEL WITH EACH INCREMENT IN THE DOSAGE OF TRIAC OR THYROID

Triac			Thyroid		
Daily dose*	Change in BMR (%)	Change in serum cholesterol (%)	Daily dose*	Change in BMR (%)	Change in serum cholesterol (%)
0.5 mg. [4]	4.4	24.3	15 mg. [2]	17.3	23.3
1.0 mg. [5]	9.8	34.7	30 mg. [4]	22.7	23.8
1.5 mg. [3]	9.1	34.2	45 mg. [2]	26.5	35.8
2.0 mg. [4]	12.8	38.3	65 mg. [4]	34.5	42.5
3.0 mg. [3]	30.3	42.3	100 mg. [3]	47.9	51.0
4.0 mg. [1]	43.5	37.5	130 mg. [3]	57.4	42.4

* The numbers in brackets refer to the number of patients tested at each dose level.

of Triac fall on a curvilinear line (Fig. 4) which demonstrates very little change in the first half of the curve, with the greatest portion of the change in the latter half. This curve may be described by the equation $y = kd^n$, where d is the dose and n is greater than unity. However, the BMR cannot increase indefinitely. It must approach a limiting value in the higher dose range, or the patient would die.

When the percentage changes in BMR with increasing doses of thyroid substance are plotted (Fig. 4), the data describe a fairly good straight line. It has previously been shown by Means and Lerman (10) that thyroxine decay is represented by an exponential equation of the form $y = C + Ae^{-kt}$, where t is the time factor, C is the limiting value which y approaches, and A is the increment of metabolism between the value of y at

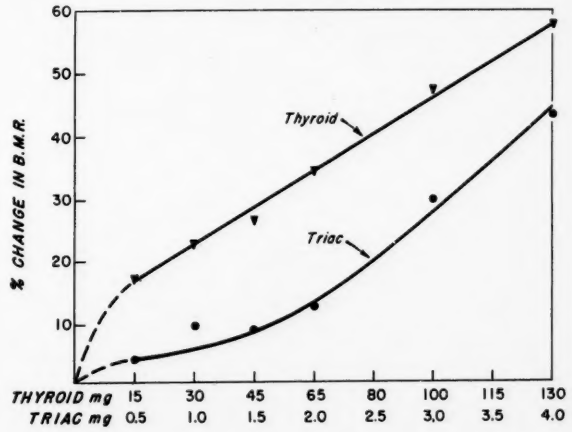


FIG. 4. The percentage change of the BMR in patients with myxedema receiving graded dosages of thyroid and Triac.

its origin and the limiting value C. Since the degree of response is proportional to the dosage, then one might expect that the dose-response curve might also be described by an exponential equation. Undoubtedly many factors determine the nature of this curve. In any case, the increase in BMR must approach a limiting value, unless death ensues.

The data for the changes in serum cholesterol produced by graded doses of Triac or thyroid do not fall on a simple curve or straight line, probably because of the limited number of observations and the large technical error inherent in the determination of cholesterol.

The dissociation of the BMR response from the cholesterol response, as produced by Triac and Tetrac, has also been reported for other deaminated thyronines such as the propionic acid derivatives (8, 9). In our experience the propionic acid derivative of thyroxine in dosages of 10 to 12 mg. daily produced a standard metabolic and cholesterol response; in dosages of 4 to 5 mg. daily there was no metabolic response, but a moderate drop in the serum cholesterol level. The propionic acid derivative of triiodothyronine in dosages of 2 to 3 mg. daily produced a standard response in both BMR and serum cholesterol concentration. As yet we have had no experience with smaller doses of this analogue.

The formic acid derivative of thyroxine, in dosages up to 20 mg. daily, failed to affect the BMR or the serum cholesterol level.

The dissociation between the response of the metabolism and that of the serum cholesterol to the administration of Triac in patients with myxedema raised the possibility of its use in the management of cases of myxedema with cardiac complications. Actually 2 of the 5 patients treated with Triac had angina. During the course of their treatment with Triac there was definite amelioration of the severity and frequency of the angina. None of the patients treated with thyroid had angina before treatment nor did angina develop during treatment. However, in the past we have observed patients with myxedema and angina who experience marked improvement in the angina as the dosage of thyroid was increased slowly from an initial dose of 15.0 mg. to a level of 45.0 mg. daily.

This dissociation of the responses to Triac also raises the possibility of its use in the treatment of hypercholesterolemia in euthyroid patients. It would be highly desirable to lower the serum cholesterol level in such patients without disturbing the BMR, particularly in cases of hypercholesterolemia and heart disease. This point is now being studied by us and by several other investigators.

Acknowledgment

I am indebted to the Warner Chilcott Laboratories for a generous supply of the analogues used.

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INITIAL ELECTRICAL CAPACITY OF THE SKIN IN THYROID DISEASE

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ABSTRACT

Up to the present, 4 methods applicable to study of the electrical properties of skin have been proposed for the diagnosis of thyroid diseases: constant-current resistance, capacitance with audio-frequency current, impedance angle, and impedance. However, accurate correlation of these parameters with the histologic structure of the skin has not been possible. In this study, the initial capacity of the skin to resist a constant current was measured by a new electrical method in 75 untreated subjects with suspected thyroid disease. It was found that: 1) the correlation coefficient between the initial capacity and the basal metabolic rate was 0.90 in 19 males and 0.74 in 56 females, and between the initial capacity and the 24-hour thyroidal I^{131} uptake was 0.88 in 13 males and 0.61 in 33 females; 2) the initial capacity was normal or higher in 15 hyperthyroid patients and the lower than normal in 5 hypothyroid patients, whereas in 17 euthyroid subjects with nontoxic goiter and in 17 subjects with nonthyroid disease, it was close to the mean "normal" value; 3) in the course of the treatment of thyroid diseases, the initial capacity gradually returned to the normal level, together with the BMR; and 4) histologic examination showed that the initial capacity was negatively correlated with the thickness of the horny layer of the skin ($r = -0.94$), a finding compatible with Diehl's observation that the horny layer is thin in Basedow's disease and thick in myxedema.

ABNORMAL electrical properties of the skin have been demonstrated in diseases of the thyroid gland. Vigouroux observed the influence of the thyroid on the constant-current resistance of the skin as early as 1888. In more modern times, Lueg and Grassheim (1) in 1929, using alternating current, showed changes in the "Kondensatorwert" of the skin in thyroid diseases. In 1933 Brazier and Lond (2) introduced a technic of measuring the impedance angle of the skin as an improved test of the thyroid function. Barnett (3) in 1939 showed a marked decrease of impedance in thyrotoxicosis.

On the other hand, the thinning of the skin in thyrotoxicosis and its thickening in myxedema have long been recognized by clinicians. In 1943

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Diehl (4) demonstrated histologically that the horny layer was thin in Basedow's disease and thick in myxedema.

In the present paper, a new electrical method for measuring the initial electrical capacity of the skin is described and its diagnostic significance in thyroid diseases is suggested, based on clinical as well as histologic observations.

METHODS

The bridge circuit shown diagrammatically in Figure 1 was used. In the equivalent circuit, R exceeds r with such a great difference that R was first determined as a constant-current resistance. To the skin and the equivalent circuit, a repeated square wave pulse was applied. Both the initial capacity and the initial resistance to a constant current were then determined by adjusting c and r so that the initial current flowing from the skin and from the equivalent circuit were balanced, as observed with a cathode-ray oscilloscope. The values of the initial capacity obtained by this method apply only to a brief interval of less than 30 microseconds from the beginning of the flow of the square wave pulse. A pair of AgCl electrodes (area, 3 sq. cm.) were placed 4–5 cm. apart on the flexor side of the forearm, one lying just below the bend of the elbow. The whole test may be performed in less than five minutes.

For histologic examination, the specimens were obtained from the skin at the site of

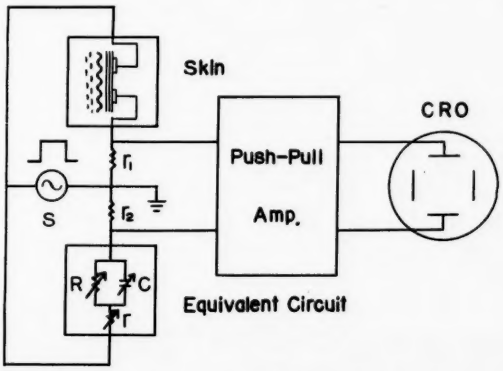


FIG. 1. Block diagram demonstrating the principle of the method.

S: Source of current. For the measurement of the initial skin capacity it operates as a pulse generator, providing square-wave pulses, 5 milliseconds, 1 volt, 50 c.p.s.; for measurement of constant-current resistance it generates a constant voltage, 1 to 10 volts. r_1. r_2: In the measurement of the initial capacity, r_1 and r_2 are each 100 Ω; in the measurement of constant-current resistance they are each 100 KΩ. Equivalent circuit: The condenser, c, and the 2 resistances, r and R are variable. Since r is always negligibly small as compared with R, R is first determined as a constant-current resistance. Then c and r are adjusted so that the initial current flowing from the skin and from the equivalent circuit are balanced, as observed with cathode-ray oscilloscope. Push-pull amplifier: Gain, 92 db; frequency characteristic, 10 kc flat, 30 kc 3 db down; discrimination factor, −90 db. CRO: Cathode-ray oscilloscope.

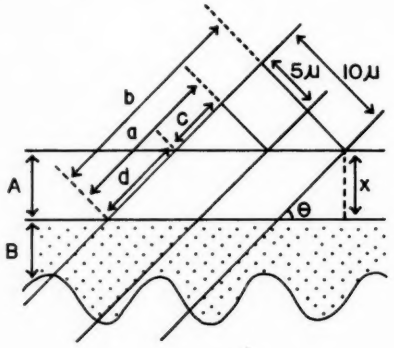
the thyroidectomy incision in patients with hyperthyroidism and nontoxic goiter. The thickness of each layer in the epidermis was measured in sectioned histologic specimens. For all tissue samples, formalin-fixation, alcohol-dehydration, paraffin-embedding and hematoxylin-eosin-staining were performed simultaneously under identical conditions. The specimens were cut in 5μ or 10μ thickness, as vertically as possible to the skin surface. In order to eliminate the error introduced by obliqueness of sectioning, successive sections of different thickness were measured, and the geometric calculation described in Figure 2 was utilized.

RESULTS

Since the distribution of the values for initial capacity in males was somewhat different from that in females, it had to be treated separately.

Correlation of the initial capacity of the skin with the BMR and the 24-hour thyroidal I^{131} uptake

In Figure 3 is shown the correlation between the initial capacity of the skin and the basal metabolic rate (BMR) in 75 untreated subjects with suspected thyroid disease (19 males and 56 females). The correlation coefficient was 0.90 for 19 males and 0.74 for 56 females. Among 46 of the 75 sub-



$$x = d \sin\theta \ \mu$$

$$b = a + c \ \mu$$

$$d = a - c = 2a - b \ \mu$$

$$\sin\theta = \frac{5}{\sqrt{5^2+(b-a)^2}}$$

$$x = (2a-b)\frac{5}{\sqrt{5^2+(b-a)^2}} \ \mu$$

FIG. 2. Calculation of the thickness of the horny layer of the skin.

a: measured thickness of the horny layer in a section of 5μ thickness. **b**: measured thickness of the horny layer in a section of 10μ thickness. In both these measurements an initial focus was made at the uppermost junction of the epidermis and dermis and a reading taken on the ocular micrometer. Then, by movement of the fine adjustment screw, a focus was made on the deepest edge of the outer surface of the epidermis. The differences between these settings on the micrometer scale are **a** and **b**. **x**: actual thickness of horny layer in the specimen. **c** and **d** are calculated distances, indicated in the figure, and useful only in the mathematical derivation of true thickness. θ is the angle of section at the surface of the skin. The thickness, **x**, of the horny layer was obtained by the method of calculation in the figure.

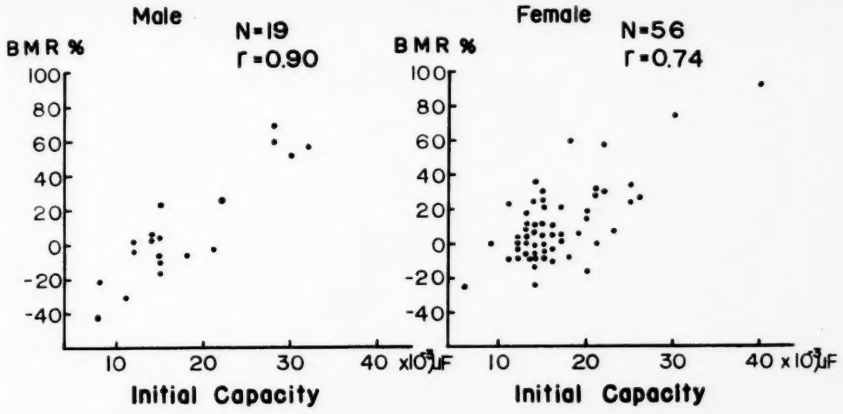


FIG. 3. Correlation between the initial skin capacity and the BMR.

jects in which 24-hour I^{131} uptakes were calculated (13 males and 33 females), the correlation between the initial skin capacity and the 24-hour thyroidal I^{131} uptake was 0.88 in the males and 0.61 in the females, both values being statistically significant ($P < 0.01$).

Relationship of the initial capacity of the skin to the functional state of the thyroid gland

In 37 of the 75 subjects the thyroid states (adequately diagnosed from both clinical symptoms and laboratory data such as BMR and I^{131} uptake) were divided into three categories: hyperthyroidism, hypothyroidism and euthyroid state with goiter. As shown in Table 1, all 5 cases of hypothyroidism with a BMR below −20 per cent and a 24-hour I^{131} uptake below 11 per cent showed low values for initial skin capacity (6 to $11 \times 10^{-3} \mu F$). On the other hand, 15 cases of hyperthyroidism showed higher values of initial capacity (14 to $40 \times 10^{-3} \mu F$) than the cases of hypothyroidism. In Table 2 an approximate parallelism is shown between the severity of the

TABLE 1. INITIAL ELECTRICAL CAPACITY OF THE SKIN IN HYPOTHYROIDISM

Case No.	Patient	Sex & age (yrs.)	Skin roughness	Serum cholesterol (mg./100 ml.)	BMR (%)	Thyroidal 24-hour I^{131} uptake (% dose)	Initial skin capacity ($\times 10^{-3}$ μF*)
1	H.R.	F 34	+	295	−25	2.5	6
2	O.K.	M 52	+	271	−41	2.0	8
3	N.Y.	M 26	+	274	−24	3.2	8
4	O.Y.	F 57	+	395	−28	8.8	10
5	M.S.	M 48	+	278	−40	10.8	11

* Microfarad.

TABLE 2. INITIAL ELECTRICAL CAPACITY OF THE SKIN IN HYPERTHYROIDISM

Case No.	Patient	Sex & age (yrs.)	Palpitation	Exophthalmos	Goiter	BMR (%)	Thyroidal 24-hour I^{131} uptake (% dose)	Initial skin capacity ($\times 10^{-3}$ μF)
1	O.R.	F 22	+	−	+	37	48	14
2	T.S.	F 32	+	−	±	60	47	18
3	S.S.	F 33	+	−	+	32	57	21
4	S.T.	F 31	+	+	+	0	91	21
5	H.K.	F 36	+	+	+	28	65	21
6	I.S.	F 24	−	+	+	30	62	22
7	Y.Y.	F 43	+	+	+	58	52	22
8	K.M.	F 22	+	+	+	26	64	26
9	O.M.	M 51	+	−	±	69	72	28
10	N.S.	M 34	+	±	+	59	59	28
11	Y.M.	M 28	+	−	+	52	72	30
12	U.T.	F 24	+	+	+	24	67	30
13	M.H.	M 29	+	−	+	57	66	32
14	Y.S.	F 53	+	+	+	51	60	36
15	I.S.	F 34	+	+	+	92	64	40

disease and the value for initial skin capacity. In 17 euthyroid subjects with goiter, as well as in 17 subjects with nonthyroid disease (diagnosed from both clinical symptoms and laboratory data), the initial skin capacity was in the range of 11 to $23 \times 10^{-3}\mu$F, as shown in Tables 3 and 4.

Changes in the initial capacity of the skin in the course of treatment of thyroid disease

The changes of the initial skin capacity in the course of treatment were

TABLE 3. INITIAL ELECTRICAL CAPACITY OF THE SKIN IN EUTHYROID STATES WITH GOITER

Case No.	Patient	Sex & age (yrs.)	Palpitation	Exophthalmos	Goiter	BMR (%)	Thyroidal 24-hour I^{131} uptake (% dose)	Initial skin capacity ($\times 10^{-3}$ μF)
1	T.H.	F 59	−	−	+	23	6	11
2	Y.H.	F 49	+	+	+	− 1	9	12
3	H.M.	F 17	−	−	+	− 2	32	12
4	K.T.	F 17	−	+	+	1	5	12
5	K.M.	F 35	+	−	+	12	27	13
6	I.S.	F 34	+	−	+	3	25	13
7	M.E.	M 19	−	−	+	2	50	14
8	S.S.	M 25	−	−	+	4		15
9	T.N.	F 45	−	−	+	− 3	13	15
10	M.M.	F 52	−	−	+	− 3	17	16
11	K.H.	F 22	−	−	+	−11	13	16
12	U.K.	M 17	+	−	+	6	18	17
13	O.M.	F 18	−	−	+	5		17
14	M.E.	F 41	−	−	+	2	17	17
15	S.T.	F 23	+	−	+	17	26	20
16	S.Y.	M 28	−	−	+	− 3	15	21
17	S.M.	F 22	−	−	+	8	20	23

followed in 3 patients with hypothyroidism and in 3 patients with hyperthyroidism. As shown in Figure 4, the initial capacity gradually returned to the normal range, together with the BMR.

Correlation between the value for the initial electrical capacity and the thickness of the horny layer of the skin

In order to find an organic basis for the significant correlation between the initial skin capacity and thyroid function in patients with nontoxic goiter or thyrotoxicosis, the initial capacity was measured on the skin over

TABLE 4. INITIAL ELECTRICAL CAPACITY OF THE SKIN IN NONTHYROID DISEASES

Case No.	Patient	Sex & age (yrs.)	BMR (%)	Skin	Initial skin capacity ($\times 10^{-3}$ μF)	Diagnosis
1	I.S.	M 55	+ 2	rough	11	Diabetes mellitus
2	N.K.	M 46	+ 2	rough	12	Lipodystrophy
3	Y.T.	M 46	− 4	rough	12	Nephrosis
4	T.E.	F 39	− 6	normal	13	Dystonia vegetatiba
5	I.M.	F 26	− 8	normal	14	Myasthenia gravis
6	S.N.	F 43	− 6	normal	14	Neuroses
7	S.Y.	F 15	−24	normal	14	Anorexia nervosa
8	T.S.	F 30	− 1	normal	14	Multiple sclerosis
9	M.K.	F 30	+ 7	normal	14	Malignant hypertension
10	S.Y.	F 21	+12	normal	15	Neuroses
11	K.Y.	M 26	−11	normal	15	Diabetes insipidus
12	M.A.	M 41	−17	normal	15	Atrial fibrillation
13	R.K.	M 51	+21	normal	16	Polyneuritis
14	M.M.	F 18	− 9	normal	18	Neurosis
15	N.K.	M 20		normal	20	Acromegaly
16	I.K.	F 58		normal	21	Hematomyelia
17	K.R.	M 32	+26	normal	22	Chronic leukemia

the thyroid gland and was related to the histologic measurements of thickness in the skin specimen taken from the same area at the time of operation. The measurement of the thickness of the horny layer and the noncornified layer as a whole was carried out at several different sites, and the mean value was corrected by the method described in Figure 2. Statistical analysis showed a significant negative correlation between the initial capacity and the thickness of the horny layer as shown in Figure 5 ($r = -0.94$, $P < 0.01$). On the other hand, no clear correlation was demonstrated between the initial capacity and the thickness of the noncornified layer as a whole ($r = 0.02$, $P < 0.05$).

By repeated stripping of the horny layer of the skin with adhesive tape, the initial electrical capacity gradually increased, confirming the negative correlation between the initial capacity and the thickness of the horny layer. For instance, by repeating the process of attachment and removal of

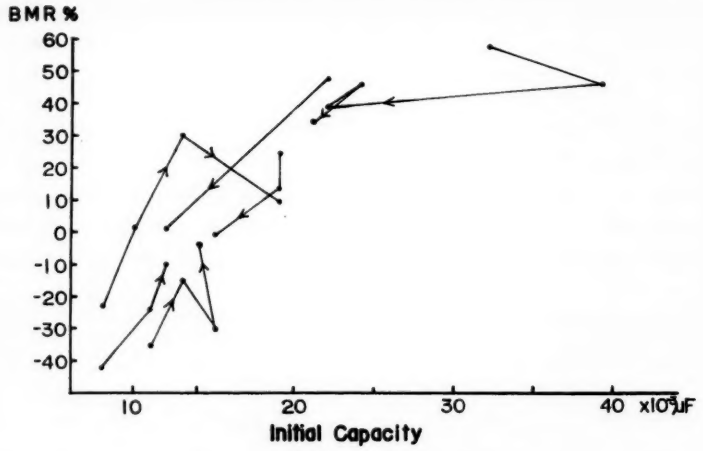


FIG. 4. Changes in the initial skin capacity during the course of treatment. Arrows indicate the direction of the change.

the adhesive tape 16 times, the initial capacity gradually increased from 13 to $40\times10^{-3}\mu$F.

DISCUSSION

If the histologic structure of the skin in thyrotoxicosis, as demonstrated by Diehl (4), could be correlated with certain electrical properties, then a new and reliable method for the diagnosis of thyroid diseases would be available. Up to the present, 4 electrical methods applicable to studies of

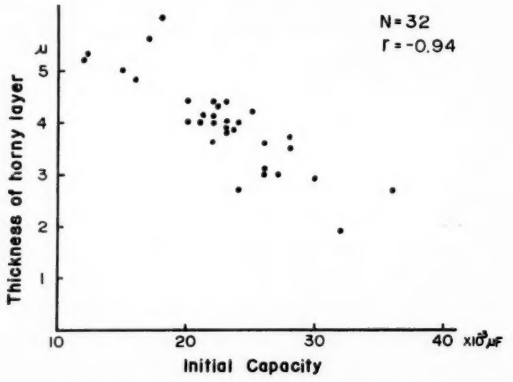


FIG. 5. Correlation between the initial electrical capacity and the thickness of the horny layer of the skin.

the skin already have been proposed for the diagnosis of thyroid diseases, namely, constant-current resistance, capacitance with audio-frequency current, impedance angle, and impedance. As Thomas and Korr (5) have shown, however, the constant-current resistance of the skin usually fluctuates with the change of sweat gland activity. The capacitance of the skin for audio-frequency current is inversely proportional to the frequency of the current oscillations. Neither the impedance angle nor the impedance reflects the electrical properties of the skin itself, because the values obtained include the impedance of deeper tissues as well. These 4 electrical properties, therefore, cannot be correlated accurately with the histologic structure of the skin.

The question has remained open as to which integumentary structure accounts for the initial capacity to resist a constant current. Hozawa (6) claimed that the horny layer is the site of the initial capacity. He based this conclusion on his own electrical experimental data, but he presented no histologic data. Ebbecke (7), on the other hand, thought that this property is localized in a single surface between the stratum lucidum and stratum granulosum. In the present study, a significant negative correlation was established between the initial electrical capacity of the skin and the thickness of the horny layer, as shown in Figure 5. No correlation could be detected between the initial capacity and the thickness of the noncornified layers. Furthermore, by progressively stripping the horny layer by repeated application and removal of adhesive tape in an exploratory experiment, readings of gradually increasing initial capacity were obtained. Therefore, it seems reasonable to conclude that the initial capacity of the skin to resist a constant current is intimately related to the thickness of the horny layer itself, and that the higher the initial capacity, the thinner the horny layer.

As shown in Figure 3, however, there was a significant positive correlation between the initial capacity and BMR.

In a previous paper (8) it was shown that the average initial electrical capacity of the skin of normal subjects was $19.5\times10^{-3}\mu$F with a standard deviation of 3.5×10^{-3} in 57 males, and $18.0\times10^{-3}\mu$F with a standard deviation of 3.2×10^{-3} in 32 females. Hence a range of 12.5×10^{-3} to $26.5\times10^{-3}\mu$F in males, or a range of 11.6×10^{-3} to $24.4\times10^{-3}\mu$F in females, will include 95 per cent of normal values (average, ±2 S.D.). In Tables 1–4 the data show that the initial capacity is low in hypothyroidism, normal or high in hyperthyroidism, and normal in the euthyroid state as well as in nonthyroid diseases.

The thinning of the horny layer in hyperthyroidism and its thickening in hypothyroidism have thus been demonstrated electronically, in striking agreement with Diehl's observations (4). Furthermore, the significance of

these observations has been confirmed by the return of the initial skin capacity to normal in the course of the treatment of thyroid disease.

From these results, it might be suggested that measurement of the initial electrical capacity of the skin would provide a reliable aid in the diagnosis of thyroid diseases when the initial capacity exceeds the normal range. The advantages of this method are the brevity of the procedure and the lack of need for any special prior preparation. However, in view of the considerable overlap between individual values in different states of thyroid function (Tables 1–3), the application of this method for the diagnosis of thyroid diseases requires careful analysis.

Acknowledgment

We are indebted to Dr. Tsutomu Wakabayashi, Professor of Physiology, University of Tokyo School of Medicine, for valuable advice and support throughout this work.

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COMPARATIVE ACTIVITIES OF TWO GONADOTROPIN PREPARATIONS FROM THE URINE OF POSTMENOPAUSAL WOMEN*

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ABSTRACT

Two preparations of human gonadotropin (HPG) from postmenopausal urine—HMG-J5 and Pergonal—extracted and purified by a chromatographic method employing zeolite and Permutit as the adsorbent material, respectively, were tested in 4 bio-assay systems. It was found that the activity contained in 1 mg. of HMG-J5 was somewhat greater than that contained in 1 mg. of Pergonal. Seven assays were performed; the mean relative potency of Pergonal in terms of HMG-J5 was 0.68. The biologic activities of the 2 preparations studied were similar as judged by Gaddum's index of discrimination, which did not differ significantly from unity for all combinations of assays.

TWO preparations of urinary postmenopausal gonadotropic material (HPG) named HMG-J5 and Pergonal have been compared by Albert and Derner (1).

Their studies showed that these 2 preparations did not differ biologically in their assay system, which involves the simultaneous response of the ovary and the uterus of the immature rat.

The present study was undertaken to investigate further the question of the biologic similarity of HMG-J5 and Pergonal. For this purpose both preparations were subjected to simultaneous comparisons in several biologic assay systems. Quantities of HMG-J5 and Pergonal, sufficient to carry out this work, were generously supplied by Dr. S. G. Johnsen, Copenhagen, Denmark and Dr. P. Donini, Rome, Italy, respectively.

MATERIAL AND METHODS

Material

HMG-J5 was extracted and purified by a chromatographic method from postmenopausal urine; zeolite was used as the adsorbent material (2). Pergonal was similarly extracted and purified by a chromatographic method, except that Permutit was used as the adsorbent material (3).

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Methods of assay

Mouse uterine weight assay. Immature white Swiss albino female mice (each weighing 8–10 Gm.) were obtained from Armer Enterprises, Croton Falls, New York. Three doses of HMG-J5 (18 μg., 36 μg., and 72 μg. per mouse) and three doses of Pergonal (20 μg., 40 μg., and 80 μg. per mouse) were given to 6 groups of mice. The seventh group served as controls and received the injection vehicle.

The materials were administered in distilled water. Regardless of the dose of extract administered the solution was such that the total volume of fluid injected into each mouse was 2.5 ml. The total dose was divided into five single subcutaneous injections of 0.5 ml. each, given during the course of three days. Autopsies were carried out about seventy-two hours after the first injection. The uteri were cut out at the insertion of the oviducts and immediately distal to the cervix uteri; they were dissected, pressed between filter papers and weighed at once on a Roller-Smith torsion balance.

Ovarian weight assay in immature female rats treated with human chorionic gonadotropin (HCG). Immature Sprague-Dawley female rats obtained from Madison, Wisconsin, were used. Two doses of HMG-J5 (175 μg. and 350 μg. per rat) and two doses of Pergonal (175 μg. and 350 μg. per rat) were given to 4 groups of rats. A dose of 20 I.U. of HCG was simultaneously injected by the same route into each animal. A fifth group served as controls and received only 20 I.U. of HCG.

The materials were administered in distilled water. Regardless of the dose of extract administered, the total volume of fluid injected into each rat was 3 ml. The same volume was used to administer the total dose of HCG assigned to each animal. The total dose was divided into six single subcutaneous injections of 0.5 ml. each, given during the course of three days. Autopsies were carried out about seventy-two hours after the first injection. Ovaries were removed, freed from fat and fibrous tissue, pressed between filter papers and immediately weighed on a Roller-Smith torsion balance.

Ventral prostate and testicular weight assay in immature hypophysectomized male rats. Immature Sprague-Dawley hypophysectomized male rats obtained from Hormone Assay Laboratories, Chicago, Illinois were used. Two doses of HMG-J5 (125 μg. and 250 μg. per rat) and two doses of Pergonal (125 μg. and 250 μg. per rat) were given to 4 groups of animals. A fifth group served as controls and received the injection vehicle.

The extracts were administered in distilled water beginning on the third day after hypophysectomy. Regardless of the dose of material administered, the solution was such that the total volume of fluid injected into each mouse was 4 ml. The total dose was divided into eight single subcutaneous injections of 0.5 ml. each, given during the course of four days. Autopsies were carried out about ninety-six hours after the first injection. The ventral prostate and testes were removed, freed from fat and fibrous tissue and immediately weighed on a Roller-Smith torsion balance.

Statistical analysis. Statistical evaluation of the data was conducted according to the methods of Fisher (4), Bülbring (5), Bliss (6) Emmens (7) and Gaddum (8). In each end-point, the first dose of each of the two extracts used produced a response differing from the corresponding mean control value by three times its standard deviation.

RESULTS

The bio-assay data are summarized in Table 1. Examination of replicate assays for each of the end-points used indicates good agreement between the individual values of relative potency as expressed by the variance ratio (F). Furthermore, there was complete overlap in confidence limits, that is,

TABLE 1. ASSAY OF PERGONAL IN TERMS OF HMG-J5

End-point	N	Design	λ	Significance of differences of slopes t (P)	R.P.	Confidence limits, P:0.95	Mean R.P.	F (P)
Uterine weight (immature mice)	42	3×3	0.22	0.04 (>0.5)	0.72	0.53–0.98	0.76	0.328 (>0.5)
	35	3×3	0.11	0.04 (>0.5)	0.80	0.67–0.95		
Ovarian weight (HCG-treated rats)	18	2×2	0.16	0.01 (>0.5)	0.51	0.36–0.72	0.59	0.978 (>0.05<0.5)
	20	2×2	0.13	0.01 (>0.5)	0.69	0.53–0.90		
Testicular weight (hypophysect'd rats)	19	2×2	0.15	0.01 (>0.5)	0.60	0.44–0.82	0.61	0.012 (>0.5)
	16	2×2	0.20	0.01 (>0.5)	0.62	0.39–0.98		
Ventral prostate weight (hypophysect'd rats)	16	2×2	0.20	0.07 (>0.5)	0.91	0.57–1.44	0.91	

N = number of animals. Design = number of dose levels of unknown and standard.
λ = index of precision. R.P. = relative potency, or units per mg., 1 unit being the activity of 1 mg. of HMG-J5. The means are geometric means of the replicate results.

$$F = \frac{\text{Mean observed variance (within assay variation)}}{\text{Mean predicted variance (between assay variation)}}.$$

the upper confidence limits were not less than the lower confidence limits of the 2 assays on each end-point. The index of precision for the 7 assays varied from 0.11 to 0.22 and there was no evidence of significant slope differences in any of the assays ($P > 0.5$).

Table 2 shows the index of discrimination for all combinations of assays.

The statistical significance of the index of discrimination was tested by means of ordinary t tests dividing the difference of two log relative potencies (R.P.'s), each based on one or two estimates by the square root of the sum of the squared variances of the respective log R.P.'s (6). It can be seen that the index of discrimination for all combination of assays did not differ significantly from unity.

TABLE 2. INDICES OF DISCRIMINATION FOR ALL COMBINATIONS OF ASSAYS

Combination of assays	Index of discrimination (P)
Uterus (mouse) Prostate (rat)	0.84 (>0.5)
Uterus (mouse) Testes (rat)	1.25 (>0.1<0.5)
Uterus (mouse) Ovary* (rat)	1.29 (>0.1<0.5)
Testes (rat) Prostate (rat)	0.67 (>0.1<0.5)
Ovary* (rat) Testes (rat)	0.97 (>0.5)
Ovary* (rat) Prostate (rat)	0.65 (>0.1<0.5)

* HCG-treated rats.

COMMENT

In the studies reported here, the 2 preparations of HPG obtained from postmenopausal urine—HMG-J5 and Pergonal—were compared in 4 assay systems: 1) the mouse uterine weight assay, which presumably measures total gonadotropic activity; 2) the augmentation assay (HCG-treated female rats), which supposedly measures FSH activity; 3) the ventral prostate assay which supposedly measures LH activity; and 4) the testicular weight assay, which has been considered by some workers (9) to be specific for FSH, but more likely includes both factors (10).

All assays were valid with regard to the tests applied; in particular, there was no evidence of significant slope differences in any of the assays. The recommended test for heterogeneity of replicate assays was applied to the data (6–11). The X^2 values obtained showed that the estimated relative potencies were homogeneous ($P > 0.05$). The estimation of relative potencies revealed that the activity contained in 1 mg. of HMG-J5 was somewhat greater than that contained in 1 mg. of Pergonal.

In order to investigate whether the biologic activities contained in these 2 preparations were similar, Gaddum's (8) index of discrimination was applied to the data. This index is calculated by dividing the mean potency ratio in one test by the corresponding figure in another. It is close to unity when two substances possessing identical biologic activity are assayed, and when inert material present in the two preparations does not seriously interfere with the estimate of relative potency.

Statistical analysis showed that this index did not differ greatly from unity for all combinations of assays.

It could be concluded that HMG-J5 and Pergonal (HPG postmenopausal materials prepared from urine by two large-scale extraction methods), although somewhat different quantitatively on a weight basis, are qualitatively similar when assayed in the 4 end-points used in this study.

Acknowledgment

Acknowledgment of devoted technical assistance is made to Mr. Bernard Harring and Mr. William Lewis.

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EFFECT OF A SINGLE INJECTION OF HUMAN PITUITARY FOLLICLE-STIMULATING HORMONE ON URINARY ESTROGENS AND THE VAGINAL SMEAR IN AMENORRHEIC WOMEN

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ABSTRACT

The effect of a single injection of human pituitary follicle-stimulating hormone (HPFSH) with or without the subsequent administration of repeated doses of human chorionic gonadotropin (HCG) was studied in 5 amenorrheic women. Administration of a single dose of HPFSH resulted in each case in a significant increase in urinary estrogen excretion. When HCG was administered forty-eight hours following injection of HPFSH, there was an additional pronounced rise in urinary estrogen excretion and a concomitant increase in pregnanediol excretion. No such rise occurred when the interval between HPFSH and HCG administrations exceeded ninety-six hours. A study of the vaginal smear indicated that identical regressive changes occurred both when estrogen secretion decreased or when it was unchanged or even increased but was accompanied by an increased progesterone production. During the period of estrogen (but not of progesterone) secretion, both the karyopyknotic and eosinophilic indices showed a highly significant correlation when plotted against the logarithm of the sum of urinary estrone+17β-estradiol+estriol.

IN A previous paper the effect of repeated injections of a human pituitary follicle-stimulating hormone preparation (HPFSH) in amenorrheic women was described (1). This treatment resulted in polycystic enlargement of the ovaries, associated with a highly elevated excretion of urinary estrogen. The stimulation of the ovaries by this hormone was in most cases excessive, surpassing many times the normal ovarian reaction to endogenous pituitary stimulation.

In an attempt to obtain an effect which resembles more closely that occurring under physiologic conditions, in the present study a single injection of HPFSH was given to amenorrheic women. In some cases the HPFSH injection was followed after 48 to 192 hours by the administra-

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TABLE 1. SOME CLINICAL AND LABORATORY FINDINGS IN 5 WOMEN WITH AMENORRHEA—BEFORE TREATMENT

	Primary amenorrhea		Secondary amenorrhea		
	Case 1 (A.F.), hypopit. dwarf	Case 2 (G.H.)	Case 3 (I.J.), anorexia nervosa	Case 4 (I.G.), anorexia nervosa	Case 5 (M.F.), pubertas praecox
Age (yrs.)	22	24	21	18	19
Age at time of menarche	—	—	14	13	10
Duration of amenorrhea (yrs.)	—	—	2	1	½
Endometrial activity (type)	atrophic	atrophic	atrophic	atrophic	atrophic
Tot. urinary gonadotropins (mg.)	<1	<1	1–2	<2	8

tion of repeated doses of human chorionic gonadotropin (HCG). For the assessment of the hormonal effects on the ovary the following parameters were used: vaginal cytology, urinary estrogen and pregnanediol excretion, and on a few occasions endometrial biopsies. Urinary 17-ketosteroids and corticosteroids were also estimated.

Since at the start of the investigation all patients exhibited an atrophic endometrium (as judged by biopsy or the vaginal smear), it seemed possible to study the influence on the vaginal smear of endogenous estrogen both alone and in combination with endogenous progesterone, and to correlate the cytologic findings with urinary estrogen and pregnanediol levels.

MATERIAL AND METHODS

Subjects and procedure

Subjects. Five patients with amenorrhea were studied; in 2 the amenorrhea was primary and in 3 it was secondary of about one to two years' duration. Urinary gonadotropin excretion was low in 4 cases and normal in the fifth. The relevant anamnestic data are compiled in Table 1. It should be emphasized that in previous studies with repeated doses of HPFSH, polycystic enlargement of the ovaries and highly elevated urinary estrogen excretion could be elicited in all 5 patients.

Scheme of treatment. The patients were treated with a single intramuscular injection of 50 mg. of HPFSH. This was followed after an interval of three to eight days by the intramuscular administration of 6000 I.U. of HCG.[1] The properties of the gonadotropic hormones employed are described in detail in a previous publication (1).

Methods

Estrogen assays. The method of Brown (2) was used, with slight modifications (3, 4).

Pregnanediol was estimated according to the method of Klopper *et al.* (5), with the exception that the Allen (6) color correction equation was employed.

17-Ketosteroids (17-KS) were estimated according to the micromethod of Vestergaard (7) with slight modifications, including the use of the Allen (6) formula.

Corticosteroids were assayed as 17-hydroxycorticosteroids (17-OH-CS) by the method of Appleby *et al.* (8) as modified by Birke *et al.* (9).

[1] "Gonadex-Leo," manufactured by AB Leo, Hälsingborg, Sweden.

Gonadotropin assays were carried out by the uterine weight method in immature mice (*e.g.*, 10). Results are expressed as *mg. of total gonadotropin* in terms of a provisional human menopausal gonadotropin (HMG-20) standard preparation (11).

Endometrial biopsy specimens were evaluated as described in earlier reports (1, 12).

Vaginal smears were obtained daily with a wooden spatula from the posterior fornix of the vagina. A speculum was used in order to obtain the smear as free from cervical secretion as possible. The films were immediately fixed in ether and ethanol and stained according to the method of Papanicolaou (13). Two hundred cells were counted on each slide.

The hormonal activity was evaluated by the karyopyknotic index (K.I.), eosinophilic index (E.I.), and folded-cell index (F.I.). Each of these indices is defined as the ratio of the particular cells in question to all other cells present and is expressed as a percentage. Increases in folding and crowding of the cells and the appearance of basophilic cells with vesicular nuclei were considered as regressive changes. For quantitative assessment the percentage of "folded cells" (expressed as F.I.) was regarded as a criterion of the initial regression of the epithelium. Changes were registered according to the principles described by earlier investigators (*e.g.*, 14, 15).

RESULTS

Effect of a single injection of HPFSH

A single injection of 50 mg. of HPFSH produced a gradual increase in urinary estrogen excretion, as shown in Figure 1.

In 2 studies on Patient A.F. (Case 1) the urinary estrogen reached peak

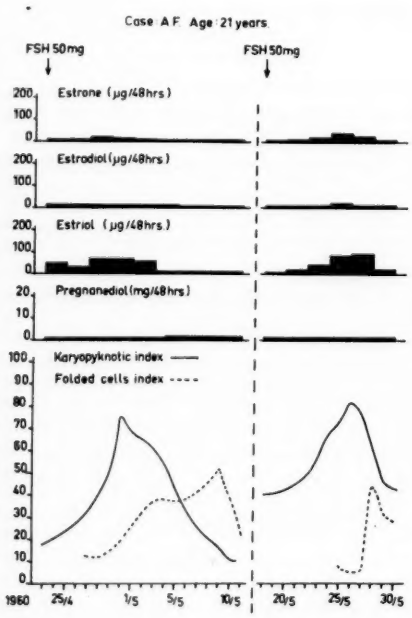

FIG. 1. *Case 1.* Urinary excretion of estrogen and pregnanediol, and karyopyknotic and folded-cell indices following a single intramuscular injection of 50 mg. of HPFSH.

values of 0.09 and 0.14 mg. per forty-eight hours, five and eight days, respectively, following the administration of HPFSH. No measurable increase occurred in pregnanediol excretion. The K.I. increased in parallel with urinary estrogens and reached peak values of 75 and 82 per cent on the fifth and eighth days, respectively. This was followed by regressive changes. No menstrual bleeding followed the administration of a single dose of HPFSH and the ovaries were not enlarged as far as this could be ascertained by pelvic examinations.

Effect of a single injection of HPFSH followed by HCG

When HCG was administered forty-eight hours after a single injection of HPFSH in Cases 1 and 2, a most marked rise in urinary estrogen and pregnanediol excretion occurred, as shown in Figures 2 and 3.

In ten and thirteen days the urinary estrogens reached maximal values of 0.7 and 1.7 mg. per forty-eight hours respectively. Polycystic enlargement of the ovaries was induced by this combined treatment. The K.I. increased rapidly, reaching values of 62 and 95 per cent in four to nine days respectively. However, the marked increase in pregnanediol excretion resulted immediately in a sharp fall in the value of K.I., which was accompanied by regressive changes (increase in F.I.). These regressive changes (occurring in the presence of very high estrogen excretion values) could not be distinguished morphologically from those found following a major decrease in estrogen excretion. In both patients, menstrual bleeding occurred approximately two weeks after the rise in pregnanediol excretion. One or two weeks later the ovaries returned to their pre-treatment size.

When the interval between HPFSH and HCG administration exceeded three days, and HCG was given 96, 120 and 192 hours, respectively, following the single HPFSH injection, this schedule of dosage did not seem to be capable of inducing ovulation. Such treatment schedules are shown in Figures 4, 5 and 6. Under these conditions, estrogen excretion was only moderately elevated; levels between 0.05 and 0.16 mg. per forty-eight hours were reached within six to seven days. The K.I. paralleled closely the estrogen excretion; values of 56 to 69 per cent were reached within the same time.

The administration of HCG did not result in any significant increase in urinary pregnanediol excretion. On the whole, the findings in these cases resembled very closely those seen following treatment with HPFSH alone (*cf.* Fig. 1). Decreasing urinary estrogen levels were associated with regressive changes in the vaginal smears. Only 1 of the 3 cases (I.G., Case 4) had uterine bleeding, approximately two weeks after the single injection of HPFSH. This was interpreted as estrogen withdrawal bleeding. In none of the patients were the ovaries enlarged.

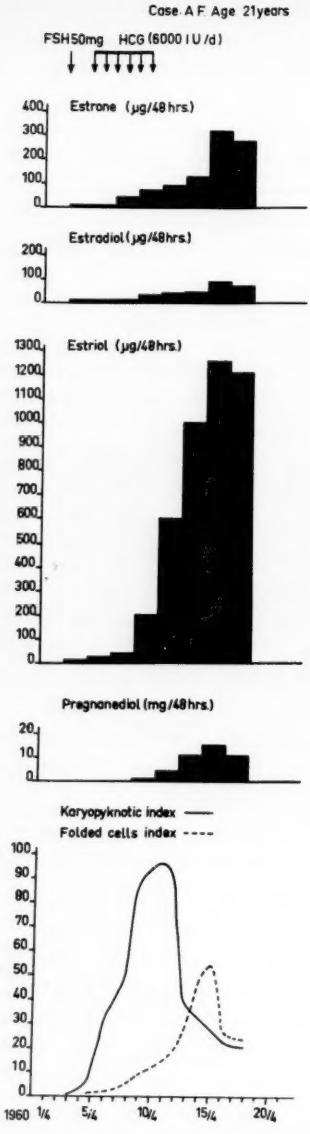

FIG. 2. *Case 1.* Urinary excretion of estrogen and pregnanediol, and karyopyknotic and folded-cell indices following a single injection of 50 mg. of HPFSH followed forty-eight hours later by repeated daily doses of 6000 I.U. of HCG.

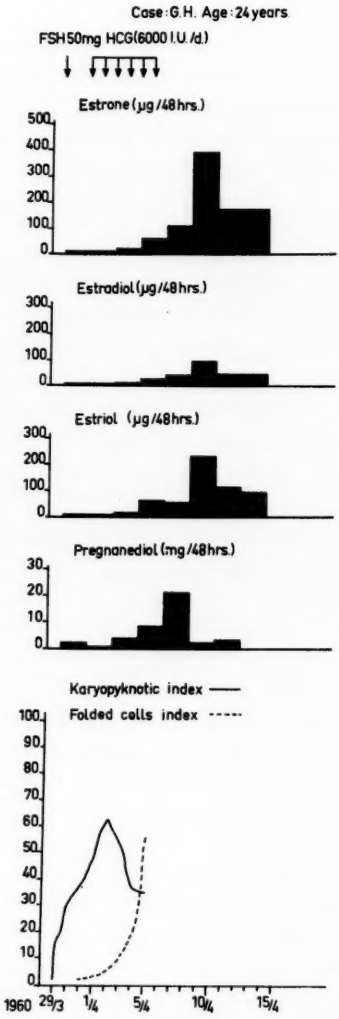

FIG. 3. *Case 2.* Urinary estrogen and pregnanediol excretion, and karyopyknotic and folded-cell indices following a single injection of 50 mg. of HPFSH followed forty-eight hours later by repeated daily injections of 6000 I.U. of HCG.

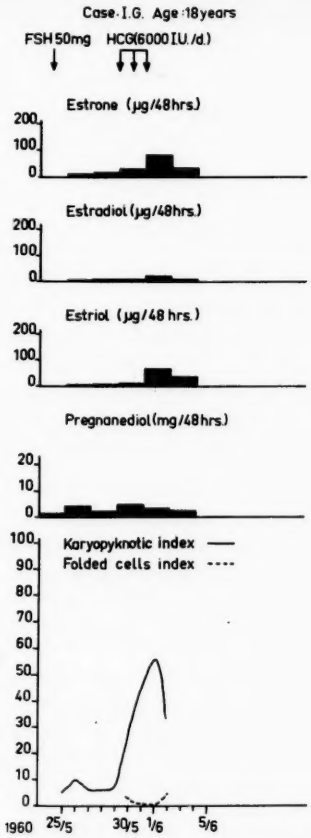

FIG. 4. *Case 4.* Urinary excretion of estrogen and pregnanediol, and karyopyknotic and folded-cell indices following a single injection of 50 mg. of HPFSH and followed ninety-six hours later by repeated daily injections of 6000 I.U. of HCG.

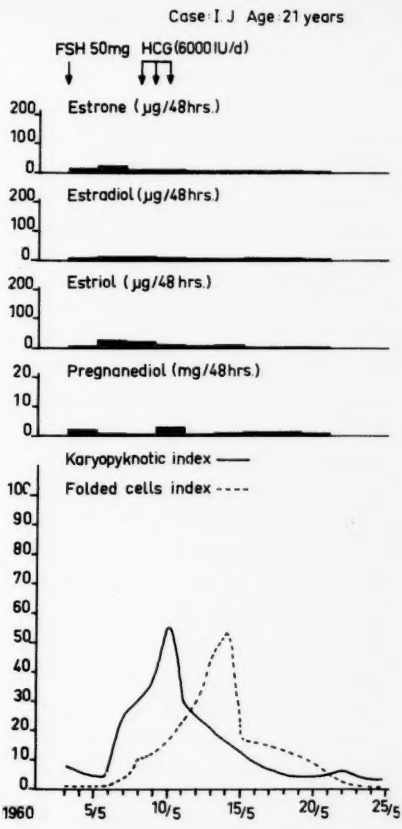

FIG. 5. *Case 3.* Urinary excretion of estrogen and pregnanediol, and karyopyknotic and folded-cell indices following a single injection of 50 mg. of HPFSH followed one hundred and twenty hours later by repeated daily injections of 6000 I.U. of HCG.

Effect on urinary 17-ketosteroids and corticosteroids

The results are summarized in Table 2. It may be seen that the period of increasing estrogen excretion was not associated with significant changes in urinary 17-KS or 17-OH-CS. The increase in 17-KS and 17-OH-CS excretion found when HCG was administered less than ninety-six hours after the single injection of HPFSH (Figs. 2 and 3) was probably due to some progesterone metabolites, as it followed closely the pregnanediol excretion.

TABLE 2. EFFECT OF A SINGLE INJECTION OF HPFSH FOLLOWED BY HCG ON THE URINARY EXCRETION OF 17-KS AND 17-OH-CS IN 48-HOUR URINE SAMPLES

	Case 1 (A.F.)			Case 2 (G.H.)		Case 3 (I.J.)		Case 4 (I.G.)		Case 5 (M.F.)	
	Before	HCG (none) (Fig. 1)	HCG (48 hrs.) (Fig. 2)	Before	HCG (48 hrs.) (Fig. 3)	Before	HCG (120 hrs.) (Fig. 5)	Before	HCG (96 hrs.) (Fig. 4)	Before	HCG (192 hrs.) (Fig. 6:
17-KS	5.0, 4.8	7.2, 6.1	12.2	14.8	36.1	13.7	11.4	17.0	19.1	14.7	23.1
17-OH-CS	9.8, 7.5	10.1, 15.1	24.2	15.0	62.5	14.2	14.9	18.8	21.1	21.4	24.4

Correlation between vaginal cytology and estrogen excretion

It was of interest to find out whether or not there was a correlation between the values for urinary estrogen excretion and those for K.I. or E.I. Since the presence of significant amounts of progesterone (or androgens) in the organism interferes with the response of the vaginal epithelium to estrogen, a prerequisite for a valid assessment of such a correlation is a virtually unchanged elaboration of progesterone (and/or of androgens and corticosteroids) in the presence of increasing amounts of estrogen produced. It was assumed that practically unchanged pregnanediol and 17-ketosteroid excretion levels could be used as parameters indicating that this criterion was met. Therefore the elevated estrogen excretion levels observed in the 5 patients in the presence of unchanged 17-ketosteroid and pregnanediol excretion were combined and their logarithms were plotted against the corresponding values of K.I., as shown in Figure 7. The data of Figure 7 indicate that there is a highly significant correlation between K.I. and the logarithm of the 48-hour excretion value (μg.) of estrone+17β-estradiol+estriol. From the equation of the line it appears that, other things being equal, a complete differentiation of the vaginal smear with a K.I. of 100 could be expected at an estrogen excretion level of 300 to 400 μg. per forty-eight hours. Such high values are never reached under normal conditions in nonpregnant women—only during pregnancy. However, in pregnancy these high estrogen values are associated with elevated pregnanediol values. Thus it would appear that a complete differentiation of the vaginal smear (with a K.I. of 100) is not a physiologic proposition in the human female.

It was also of interest to find out whether the K.I. is more closely correlated with urinary estrogen excretion than is the E.I. According to current concepts, K.I. represents a somewhat more sensitive and more reliable index of response to estrogen than does E.I. (16). Therefore the values for E.I. determined from the same smears were plotted against the values for urinary estrogen excretion in Figure 8. In this case also there was a highly significant correlation between E.I. and log estrogen excretion. If the value of the regression coefficient (b) is divided by its error (s_b), a value of 6.67 can be obtained. A similar calculation for the data of Figure 7 gives a value of 8.97. The difference between these two values is not significant and

therefore no statement can be made as to the superiority of one index or the other. Thus—at least in the type of patients studied (characterized by the absence of cytolytic factors in the vagina)—the K.I. and E.I., respectively, are equally good indicators of increasing estrogen secretion, provided no concomitant rise in urinary pregnanediol or 17-ketosteroid excretion occurs.

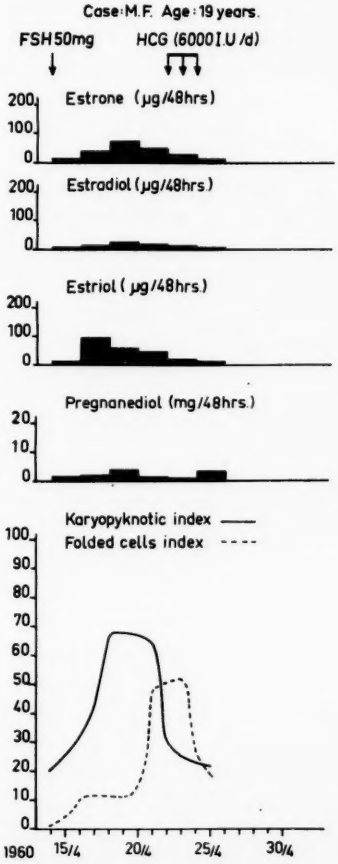

FIG. 6. *Case 5.* Urinary excretion of estrogen and pregnanediol, and karyopyknotic and folded-cell indices following a single injection of 50 mg. of HPFSH followed one hundred and ninety-two hours later by repeated daily injections of 6000 I.U. of HCG.

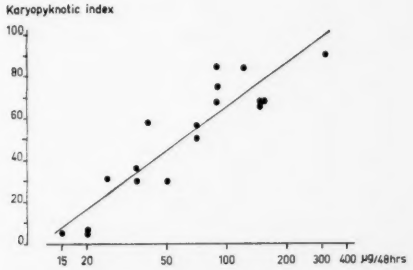

FIG. 7. Correlation between karyopyknotic index and the logarithm of the 48-hour excretion value (µg.) of estrone+17β-estradiol+estriol. The equation of the line is: $Y = -74.83 + 70.15\,x$; $(b = 70.15 \pm 7.82)$.

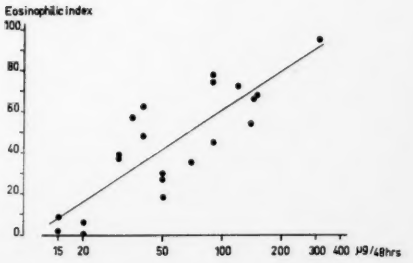

FIG. 8. Correlation between eosinophilic index and the logarithm of the 48-hour excretion value (µg.) of estrone+17β-estradiol+estriol. The equation of the line is: $Y = 66.12 + 63.27\,x$; $(b = 63.87 \pm 9.48)$.

DISCUSSION

The administration of 50 mg. of HPFSH produced in the 5 patients studied a continuous rise in urinary estrogen excretion, which reached a peak on the sixth or seventh day following a single injection. This relatively long period of increased estrogen excretion can be interpreted either by assuming that once HPFSH reaches the ovary it will induce there an increased estrogen secretion during a considerably long period, or by assuming that circulating HPFSH has a fairly long half-life and that therefore the ovaries will be stimulated continuously during a week or so. Some support in favor of the second alternative comes from a preliminary study (Diczfalusy, Gemzell and Notter, unpublished data) in which the urinary gonadotropin excretion (expressed in terms of HMG-20) was studied before and after a single injection of 50 mg. of HPFSH in 3 patients with previous implantation of Y^{90} into the sella turcica. Gonadotropically active material was excreted in the urine of all 3 patients also on the fifth and sixth postinjection days. It seems, therefore, that following a single injection of HPFSH, urinary estrogens will increase as long as biologically active HPFSH is present in the circulation, and that estrogen excretion will decrease more or less immediately following the disappearance of the hormone from the body fluids.

When HCG was administered within forty-eight hours following the single injection of HPFSH, this combined treatment caused a most marked additional increase in estrogen excretion, accompanied by a conspicuous rise in pregnanediol excretion. Ovarian size also was considerably increased. Since in these cases the increase in urinary estrogen excretion continued fourteen days or more following cessation of HPFSH therapy, *i.e.*, during a period when presumably no HPFSH was circulating in the body, it seems reasonable to assume that this additional estrogen excretion was attributable to the secretory activity of the corpus luteum (or corpora lutea?). It would appear likely—although it remains to be demonstrated in hypophysectomized patients—that estrogen secretion by the corpus luteum is independent of HPFSH. Whether or not the action of HCG on the synthesis of estrogen and progesterone in the corpus luteum is a direct one, or is mediated by a pituitary factor (perhaps prolactin?) cannot be decided at present. Studies in hypophysectomized patients may clarify this problem.

When the interval between HPFSH and HCG administration was at least ninety-six hours, the injection of HCG did not result in increased estrogen or pregnanediol excretion, indicating that most probably no ovulation occurred. This observation is intriguing, since one would expect some HPFSH to be still circulating at this time. A tentative suggestion would

be that at the time when HCG reached the follicles, they were no longer susceptible to the luteinizing action of HCG, perhaps because of beginning atretic changes. If so, this would indicate that, in order to elicit ovulation, there must be an optimal time-interval between the actions of HPFSH and HCG. This raises the interesting question, whether or not there is a sequential release of FSH and LH from the human pituitary in the course of normal ovulation; continued studies might provide an answer.

Another aspect of the present study, namely, the correlation of vaginal cytology with urinary estrogen excretion also deserves comment. When the vaginal epithelium of the patients studied was stimulated by endogeneous estrogen alone, it reached various stages of differentiation, as indicated by the changes in K.I. and E.I. When the estrogenic stimulation caused regressive changes (such as swelling of the nuclei), folding of the cytoplasmic edges, cellular crowding and an augmentation of basophilic cells occurred. Exactly the same regressive changes were found when increasing levels of progesterone were associated with high, or even markedly increasing levels of estrogen. Therefore it must be concluded—in contrast to present concepts (*e.g.*, 15)—that by the use of the cytologic method employed here, it is not possible to differentiate between regressive changes in the vaginal smear produced by falling levels of estrogen, and those produced by an increased production of progesterone in the presence of unchanged or increased estrogen levels. It is likely that an increased production of endogenous androgen interferes in a similar manner with the assessment of estrogenic effect. Studies are in progress on this problem.

In the present study, a beginning differentiation of the cells in the vaginal smear was observed as early as one to three days following the administration of HPFSH, *i.e.*, prior to the rise in estrogen excretion. It appears therefore that very small amounts of estrogen are sufficient to induce the differentiation of the vaginal epithelial cells provided no major changes occur in the production of other steroid hormones (progesterone, androgens, and perhaps corticosteroids).

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SODIUM AND POTASSIUM METABOLISM IN A CASE OF FAMILIAL PERIODIC PARALYSIS

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ABSTRACT

In a patient with familial periodic paralysis associated with hypokalemia, sodium and potassium metabolism was studied during periods of high-sodium and low-sodium diet. Retention of sodium was common before and during the attacks, but did not appear to be an essential factor. A moderately restricted sodium diet (65 mEq. daily) failed to prevent the paralytic episodes. However, attacks seemed to be milder and less easily precipitated when dietary sodium was reduced. Electrocardiograms of this patient taken in the intervals between the attacks showed tall, narrow, peaked T-waves consistent with those ordinarily seen in patients with hyperkalemia, though concurrent serum potassium concentrations were within normal limits.

SINCE the first description of periodic paralysis by Musgrave in 1700 (1), very little was known about its pathogenesis until low serum potassium values during attacks were reported (2–4) about twenty-five years ago. An attempt was made to explain the nature of the paralytic episodes on the basis of hypokalemia, and this observation was exploited therapeutically. Attacks could be terminated by giving potassium salts by mouth; furthermore they could be induced by administering glucose, glucose and insulin, or epinephrine, all of which lower the serum potassium level (3). It was also shown that the urinary output of potassium was reduced during attacks of paralysis (4). These observations implied that, during attacks, there was a shift of potassium from the extracellular to some intracellular space. Metabolic balance studies (5) substantiated this hypothesis.

Yet familial periodic paralysis can occur without hypokalemia (6), and paralysis is frequently absent at levels of serum potassium well below those associated with paralysis in the familial syndrome. These and other discrepancies made it difficult to correlate attacks of paralysis solely with an abnormality of potassium metabolism. A new lead in the investigation of the mechanism of periodic paralysis was given by Conn and his associates (7), who reported that both spontaneous and induced attacks of periodic paralysis are preceded by a greatly increased urinary excretion of aldosterone and massive retention of sodium followed by "sequestration"

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of potassium within the body. They were unable to produce an attack with glucose and insulin when 2 patients ate a diet very poor in sodium. They (7) suggested that sodium retention is the primary factor which sets into motion the characteristic changes in potassium metabolism during an episode of periodic paralysis.

Other workers (8, 9) reported somewhat different findings. Their data suggest that although sodium retention is common during attacks of paralysis it is not invariable and not essential for paralysis. Furthermore, in 2 patients studied by Jones and his associates (9) no increase in aldosterone excretion was found before, during or after attacks of paralysis. They therefore concluded that sodium retention and increased excretion of aldosterone do not necessarily precede attacks of periodic paralysis in all cases.

This report is concerned with the investigation in another case of familial periodic paralysis of the relationship between the attacks and the retention of sodium, and the efficacy of moderate restriction of dietary sodium in the prevention of the paralytic attacks. Certain unusual electrocardiographic alterations are presented as well.

CASE REPORT

A 22-year-old soldier was admitted to the Haydarpaşa Army Hospital, Istanbul, in a state of flaccid paralysis. A history of similar spontaneous attacks of paralysis in his maternal grandfather was elicited. The patient had been in apparent good health until attacks of paralysis involving all four extremities began two months after entering the military service, nineteen months prior to his admission to the hospital. Episodes of paralysis had since been recurring one to four times a month, having their onset always in the middle of the night and usually lasting about twenty-four hours. Circumstances under which attacks occurred were not predictable.

Physical examination on admission revealed nothing abnormal except in the neuromuscular system. The blood pressure was 115/80 mm. Hg. Neurologic examination during the attack showed flaccid paralysis of all extremities and lack of tendon reflexes.

The following laboratory data were obtained during the period of observation: Hemoglobin level, erythrocyte count, leukocyte count, erythrocyte sedimentation rate and urinalysis findings were normal. The blood urea level was 38 mg. per 100 ml. A chest roentgenogram was normal. An electrocardiogram taken during an interval period, while the serum potassium level was 4.0 mEq. per liter, showed tall, narrow, peaked T-waves in precordial leads (*cf.* Fig. 2). This finding persisted in repeated electrocardiograms obtained in interval periods between the attacks.

Histologic examination of a biopsy specimen from the gastrocnemius muscle obtained during an interval period did not reveal any pathologic findings.

Urinary excretion of aldosterone was not measured.

Determinations of potassium and sodium in serum and urine were performed by flame photometer.

During the long period of observation (Fig. 1), spontaneous paralytic attacks developed every three to twenty-two days (average, every thirteen days), each lasting twenty-six to thirty hours. Attacks invariably began at night. Oral administration of 150 Gm. of glucose, with or without insulin (20 units), repeatedly induced a typical epi-

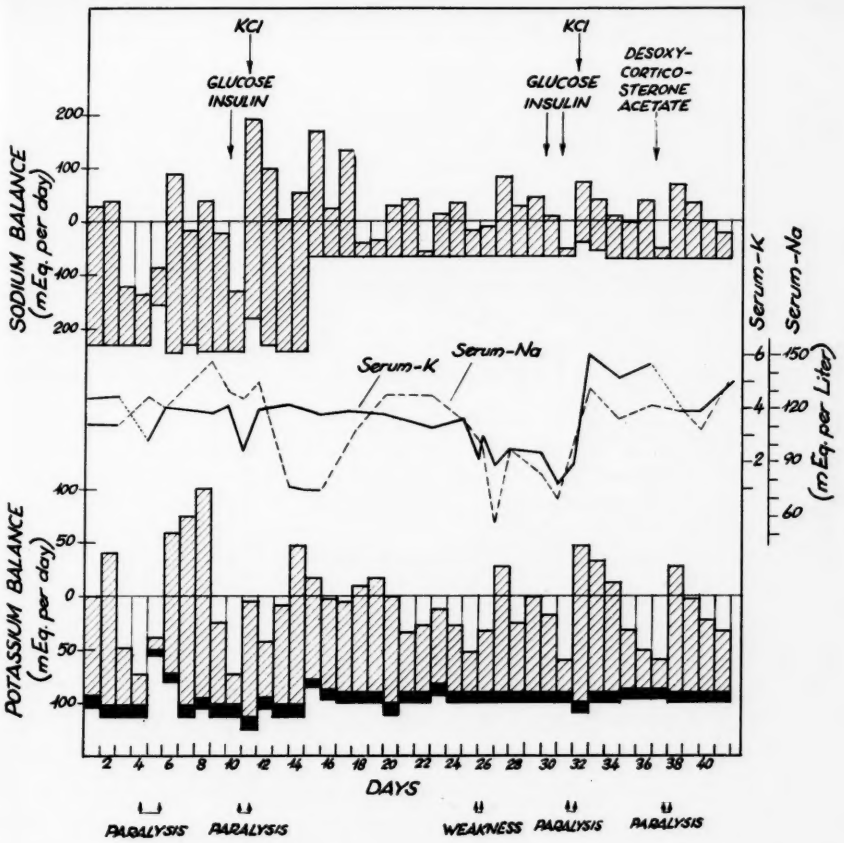

FIG. 1. Changes in sodium and potassium balances and serum sodium and potassium concentrations during period of study.

sode of flaccid paralysis. Spontaneous as well as induced attacks could be terminated within one to two hours by oral administration of 6–10 Gm. of potassium chloride.

During the period of study the patient was first given a basic diet containing 240 mEq. of sodium and 112 mEq. of potassium daily. Deviations in the intake of these substances were recorded. After fourteen days of this regimen, during which a spontaneous and an induced attack occurred, the diet was shifted to a moderately low-sodium variety containing 65 mEq. of sodium and 100 mEq. of potassium per day.[1]

With a high-sodium, high-potassium diet a spontaneous attack of paralysis developed which was clearly preceded and accompanied by massive retention of sodium (340 mEq. in three days) and of potassium (180 mEq. in three days). Serum potassium concentration fell during the attack, indicating that the retained potassium was shifted to the intracellular space (Fig. 1). Following recovery from the attack there was increased

[1] For purposes of metabolic balance, a day constituted the period from 8 a.m. till 8 a.m. the next morning.

diuresis both of sodium and of potassium, resulting in a moderately negative sodium balance and a markedly negative potassium balance. These findings were in essential agreement with those of Conn *et al.* (7).

While the patient was receiving the same diet, an attack was induced by glucose and insulin (Fig. 1). Between the induction and the onset of the attack (eighteen hours), retention of sodium and potassium was observed again, associated with hypokalemia during the period of paralysis. A pronounced negative sodium balance followed the induced attack, but no excess urinary excretion of potassium ensued.

Subsequently, during a period of low-sodium diet, an episode of muscular weakness alone was observed while the serum potassium level fell to as low as 2.3 mEq. per liter. The patient spontaneously recovered from this episode within five hours—an unusually short period in his case. This attack was preceded by retention of potassium but almost no retention of sodium, and was followed by a mild to moderate increase in diuresis of sodium and of potassium during the attack. The mild intensity and short duration of the attack despite the very low level of serum potassium is interesting and might be related to the restricted sodium intake.

On day 30 an attempt to provoke an attack by the usual agents (150 Gm. of glucose and 20 units of insulin) was unsuccessful, though the serum potassium level fell to 1.3 mEq. per liter. During this day no sodium retention occurred. It required repetition of the administration of these agents on the following day to precipitate paralysis. Once again the paralytic attack was preceded by a distinct retention of sodium and potassium and was followed by a moderately increased diuresis of both electrolytes.

Intramuscular administration of 10 mg. of desoxycorticosterone acetate on day 37 (Fig. 1) precipitated a typical attack of flaccid paralysis which was preceded by moderate retention of sodium as well as of potassium. Unfortunately, no serum potassium determinations were made during the period of paralysis. The patient recovered spontaneously within five or six hours, and a moderately increased excretion of sodium and potassium ensued.

COMMENT

Our patient was suffering from the common type of familial periodic paralysis associated with hypokalemia, and his attacks were relieved by administration of potassium. Spontaneous attacks occurred one to four times a month, usually lasting approximately twenty-four hours, with the paralysis being almost complete. Hence this case may be regarded as moderately severe.

Pertinent findings during the episodes of paralysis are summarized in Table 1. The data indicate that in this case retention of sodium and potassium preceded, and increased diuresis of sodium followed, the majority of spontaneous or induced paralytic attacks. However, with the low-sodium regimen, the total quantity of sodium retained (days 31 and 37) did not appear significantly large, and similar variations occurred at other times without being followed by an attack. Furthermore, the episode of spontaneous paresis on day 26 was not preceded by sodium retention, and the concentration of serum potassium fell to a low level of 2.3 mEq. per liter. These observations might be interpreted to mean that though retention of

sodium is common during attacks, it is not an essential feature.

A moderately restricted sodium diet such as used here failed to prevent precipitation of episodes of paralysis. Yet, the following observations might be interpreted as a favorable effect of dietary sodium restriction on the occurrence of paralytic attacks: 1) the spontaneous muscular weakness (day 26) was mild and of relatively short duration; and 2) two consecutive attempts were required to induce an attack (days 30 and 31).

In their studies, Conn *et al.* (7) were unable to produce a paralytic at-

TABLE 1. SUMMARY OF PERTINENT DATA DURING ATTACKS OF PARALYSIS

	High-sodium diet		Low-sodium diet			
	Spontaneous paralysis (day 4)	Induced paralysis (day 10)	Spontaneous paresis (day 26)	Attempted induction failed (day 30)	Induced paralysis (day 31)	Paralysis induced by DCA* (day 37)
Degree** of retention of sodium preceding attack	+++	+++	0	0	++	++
Degree of retention of potassium preceding attack	+++	+++	++	+	++	++
Serum potassium during attack (mEq./L.)	2.9	2.5	2.3	1.3	1.8	?
Degree of increased diuresis of sodium following attack	++	+++	++		++	++
Degree of increased diuresis of potassium following attack	+++	0	+		++	+

* Desoxycorticosterone acetate.
** Degrees: +++ = marked; ++ = moderate; + = mild; 0 = none.

tack by using a diet extremely poor in sodium (8 mEq. daily). De Graeff and Brocker (8) inquired whether the prevention of attacks by sodium restriction depended upon the severity of the disease. They studied a patient with periodic paralysis who had frequent severe attacks; a dietary restriction of sodium down to 10 mEq. per day did not prevent recurrence of spontaneous attacks, whereas in the patient's sister who had a mild form of the disease, attacks could not be produced when dietary salt was restricted. On the other hand, in a case of mild periodic paralysis studied by Jones and associates (9), attacks could be precipitated while the patient was receiving only 13 mEq. of sodium daily. At present, therefore, it is not clear why dietary sodium restriction seems beneficial for attacks of paralysis in some cases and not in others.

The great variability of the serum sodium level noted in the present case remains unexplained.

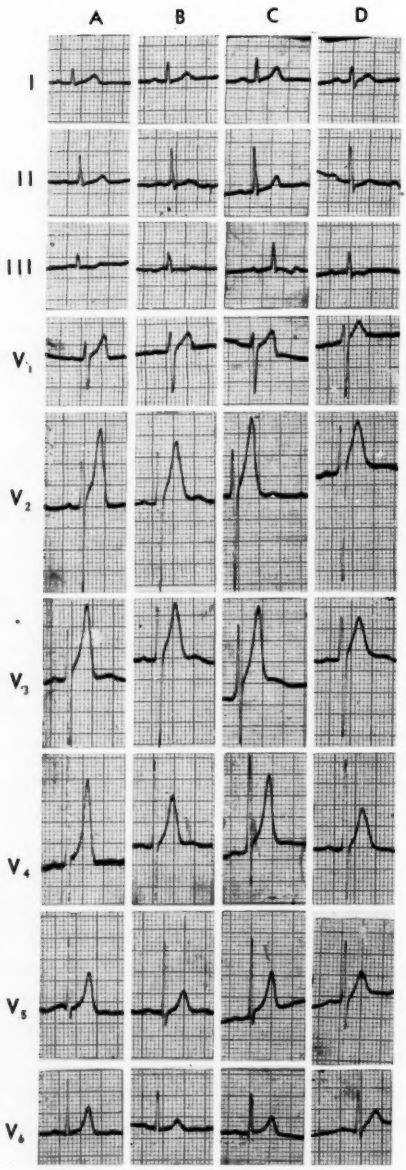

FIG. 2. Electrocardiograms of a patient with hereditary periodic paralysis showing remarkable T-wave changes. **A.** Taken on October 3, the day after a spontaneous attack of paralysis; serum K, 4.4. mEq./L.; serum Na, 121.0 mEq./L. **B.** Taken on October 7, during an induced attack of paralysis; serum K, 2.8 mEq./L.; serum Na, 111.0 mEq./L. **C.** Taken on October 11, four days after a paralytic attack; serum K, 4.5 mEq./L.; serum Na, 110.0 mEq./L. **D.** Taken on October 13, during a spontaneous attack of paralysis; serum K, 2.9 mEq./L.; serum Na, 127.0 mEq./L.

Electrocardiographic changes not commonly observed in cases of periodic paralysis occurred in our patient. As seen in Figure 2, electrocardiograms taken during the periods between the attacks demonstrated (particularly in precordial leads) tall, narrow, peaked T-waves consistent with those usually seen in patients with hyperkalemia. Hyperkalemia does not occur, as a rule, in patients with this disease, and indeed, simultaneous determinations of the serum potassium level showed it to be within normal limits. On electrocardiographic tracings obtained during the paralytic episodes, the T-waves, in becoming smaller and less peaked, approached normal contours. Yet, despite a definite decrease in the concentration of serum potassium to below 3.0 mEq. per liter, they did not show true flattening. Upon recovery from the attack, the tall, narrow and peaked T-waves reappeared. This pattern was repeatedly observed during the study of the patient.

Electrocardiographic changes ordinarily found in cases of periodic paralysis include impairment in atrio-ventricular conduction (10–12), prolonged Q-T interval (11) and flattening or inversion of the T-waves (9–12). These alterations are associated with lowering of the serum potassium level and disappear when the attack subsides, the contour of the T-waves reverting to normal. Katz (11) mentioned that T-wave changes may "overswing the mark" upon recovery from the attack but did not report the simultaneous serum potassium values. That the tall, narrow, peaked T-wave alterations in our case were not a transient "overswinging of the mark" is shown by the fact that they were present even seven days after the attack.

In relation to electrocardiographic disturbances, it is not clear whether the effects of the potassium and sodium ions are balanced against each other (11). Low serum sodium values were found concurrent with T-wave abnormalities in this case. It is possible that this change might contribute to the discordance between the contour of the T-wave and the concentration of serum potassium. It is also possible that the distribution of the potassium ion in some patients with periodic paralysis may be such that the concentrations of potassium in the serum and in the myocardial cells do not run a parallel course.

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HYPOSPADIAS IN A MALE INFANT WITH THE ADRENOGENITAL SYNDROME*

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ABSTRACT

A case of congenital adrenal hyperplasia with third-degree hypospadias is reported. Steroid excretion and testicular histology were similar to those described in the classic form of the syndrome. Because of the familial occurrence as reported from other sources, a genetic factor is assumed. Its mode of action remains unexplained.

THE presence of third-degree hypospadias and bifid scrotum in a male with congenital adrenal hyperplasia is in marked contrast with the expected finding of normal male genitalia and early penile enlargement. This report describes the physical features and the steroid excretion pattern in such a child. This child appears to be comparable to 2 male infants reported in 1941 by Werthemann (1). Autopsy showed similar genital abnormalities and, though there were no steroid studies, the clinical and pathologic picture suggested the salt-losing variety of adrenocortical hyperplasia.

CASE REPORT

M.D., a 2-month-old white male infant, was admitted to the Grace-New Haven Community Hospital on September 25, 1957. He was referred because of failure to gain weight adequately since birth, and projectile vomiting and diarrhea during the ten days preceding admission. The vomiting and diarrhea had been relieved only partially by regulation of the dietary formula and medication. He was the product of a primiparous pregnancy, and delivery had been uncomplicated. Both parents were in good health. The mother had taken no hormonal medication during the pregnancy. The family history revealed no member with genital anomalies or adrenal disease. At birth, he weighed 4200 Gm., and was noted to have an anomaly of the external genitalia compatible with a diagnosis of hypospadias. In addition, scattered areas of increased pigmentation had been found on the lateral aspect of the right thigh.

Physical examination on admission showed him to be a dehydrated, undernourished child. He weighed 4500 Gm. and measured 60 cm. in length. His temperature was 36°C., pulse rate 140, and respiratory rate 40. Skin turgor was poor, the fontanelle was sunken, and mucous membranes were dry. The lungs were clear. The heart appeared smaller than normal on percussion, and there was a soft systolic murmur. No abnormal

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masses could be palpated in the abdomen. The penis was 2 cm. long, and was encircled with normal epithelium except for the ventral surface, along which a groove extended from the tip of the glans to the base of the shaft where it met the urethral orifice at the junction of the shaft with the perineum. On either side of the base of the penis were rugose folds, each of which contained an oval, firm mass, 1.5 cm. in diameter, of testicular consistency and mobility, with cord-like attachments at the superior poles. No urogenital sinus could be found (Fig. 1 and Fig. 2).

Laboratory studies: The hemoglobin level on admission was 11.6 Gm. per 100 ml., but as hydration progressed it fell to 5.6 Gm. just prior to transfusion. The urine was clear, with no protein and no formed elements; a trace of reducing substance was noted. Initial and subsequent serum chemical values were as follows:

Date (1957)	Na (mEq./L.)	K (mEq./L.)	Cl (mEq./L.)	CO_2 (mEq./L.)	NPN (mg./100 ml.)
Sept. 25	114	9.6	85	—	58
Sept. 26	121	8.2	92	15.3	31
Sept. 28	130	6.3	101.6	18.9	—
Sept. 30	138	4.9	100	—	—
Oct. 11	136	5.1	99.6	26.6	—

Electrocardiograms were consistent with the electrolyte disturbance, and concomitantly returned to normal.

Intravenous pyelograms failed to reveal any abnormalities of the bladder and upper genito-urinary tract. X-ray examination of the wrists showed the presence of two carpal centers, consistent with the chronologic age.

The nuclear pattern of the buccal cells was repeatedly and consistently found to be chromatin-negative.

The methods used for the determination of 17-ketosteroids and pregnanetriol have been described elsewhere (2). In addition, the following procedures were carried out for further purification and identification of the isolated compounds, beginning with paper

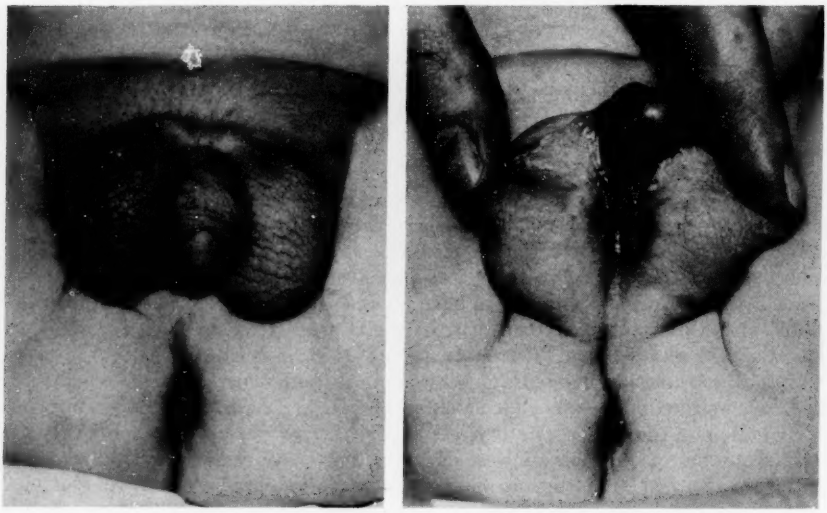

FIG. 1. Appearance of the external genitalia.

FIG. 2. Photomicrograph of a section from the testicular biopsy specimen.

chromatography (ligroin/methanol/water; 100/80/20) of the peaks containing the $C_{19}O_2$ compounds eluted from the alumina column. Dehydroisoandrosterone, androsterone and etiocholanolone were further identified by their infrared spectra. The fractions containing pregnanetriol and 11-ketopregnanetriol were oxidized with sodium bismuthate and rechromatographed on paper. Following staining with alkaline m-dinitrobenzene, a single spot was found and identified as etiocholanolone.

For the determination of urinary pregnanediol, the method of Bongiovanni and Clayton (3) was used. The results are shown in Table 1.

TABLE 1. STEROID EXCRETION IN PATIENT M.D. BEFORE TREATMENT

	Excretion (mg./24 hrs.)
Dehydroisoandrosterone	3.3
Androsterone	6.0
Etiocholanolone	2.4
11-Oxy 17-ketosteroids	4.0
Pregnanetriol	4
Pregnanediol	None
11-Ketopregnanetriol	None

DISCUSSION

The simultaneous occurrence of hypospadia, bifid scrotum, and overproduction of adrenal androgens cannot be explained in an entirely satisfactory fashion. Jost (4–5) first showed that the intra-uterine differentiation of penis and scrotum in the normal individual is induced by a substance secreted by the testes. Under pathologic conditions, *i.e.*, congenital ad-

renal hyperplasia, hormones of adrenal origin may act in a similar way, leading to the classic findings of female pseudohermaphrodism in the newborn female, or macrogenitosomia praecox in the newborn male. That this hormone is androgenic in nature is suggested by the studies of Van Wagenen and Hamilton (6) and Wells (7), and by the masculinization of female fetuses from mothers with androgen-secreting tumors (8, 9), or mothers who were given androgenic hormones during pregnancy (10–12). This aspect of ontogenesis has been reviewed by Grumbach and Barr (13), and a direct relationship between androgenic steroids and the differentiation of the external male genitalia can be assumed without much doubt.

What possible pathogenetic factors could then be implied in our patient? Deficient androgenic stimulation is unlikely. Although the problem of adrenal androgen secretion has been clarified further by the recent work of Fukushima *et. al.* (14) and Vande Wiele and Lieberman (15), the synthesis and metabolic pathways of this hormone (or hormones) in congenital adrenal hyperplasia are as yet not clearly understood (16). The fact, however, that the steroid excretion pattern in the present case did not differ significantly from that in children with the classic form of this syndrome may be interpreted as evidence against an "adrenal" etiology of the developmental defect.

The histologic changes found in the testes (Fig. 2) were similar to those described by Sobel *et. al.* (17) and Wilkins and Cara (18) in children with macrogenitosomia praecox due to adrenal hyperplasia. This in itself does not exclude a testicular etiology, since abnormal function may not be histologically detectable.

Thus at present it is not clear whether the incomplete perineal fusion is due to a deficiency of an inductor substance or to an embryologic defect. In view of the observations by Werthemann (1) and by Grumbach (personal communication) suggesting a familial occurrence of this malformation, a genetic factor may be present, although its mode of action remains obscure.

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A COMPARISON OF METHODS FOR THE ACID HYDROLYSIS OF A URINARY CONJUGATE OF ALDOSTERONE*

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ABSTRACT

A comparison was made between different methods of hydrolysis of a conjugate of aldosterone, using a common pool of urine. Following injection of 7-H^3-aldosterone into each of 4 normal women, the yield of tritium assayed specifically as aldosterone and released from the urine as the result of various hydrolytic procedures was measured. Very little difference in the yield of aldosterone was found, whether the urine was left to stand undisturbed with or without chloroform, or continuously shaken with chloroform, or continuously extracted with the same solvent in a Cohen extractor. With every procedure used, hydrolysis for 0–24 hours produced about eight times as much aldosterone as in the period 24–48 hours, and about one hundred times as much as in the period 48–72 hours. Ethyl acetate was found to be a poor solvent for the extraction of aldosterone compared with chloroform or methylene dichloride.

IT HAS been well established that an appreciable fraction of the aldosterone secreted in man is metabolized without prior alteration of the molecule (*e.g.*, reduction in ring A) to a conjugate—previously termed the 3-oxo conjugate (1–4) and in this paper referred to as a conjugate of aldosterone. Under acid conditions and at room temperature this conjugate is cleaved in the urine and aldosterone is released in the free form (1). Procedures reported in the literature (5–10) for the hydrolysis of the conjugate under acid conditions vary widely in the method of contact of the aqueous and organic phases, the length of time for which the hydrolysis is allowed to proceed, and other conditions. There is no general agreement as to which method produces the maximal yield, owing partly to lack of specificity and precision of aldosterone assays. The availability of tritiated aldosterone (11) has made possible a more accurate and specific method for the estimation of aldosterone released under various conditions. A series of experiments has been carried out on a common pool of urine, in order to

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compare directly the various methods in current use for the hydrolysis of the conjugate at pH 1.0.

MATERIAL AND METHODS

Subjects and procedure

Four normal female subjects were each given an intravenous injection of 0.1 μg. (2 μc.) of 7-H^3-*d*-aldosterone dissolved in 10 ml. of physiologic saline. The first 24-hour collection of urine from each patient was measured and made up to 2 liters with water. Equal volumes from all of these diluted urines were mixed together to form a common pool.

Neutral extract

The free aldosterone was first removed from all aliquots of urine by extracting with $1\times\frac{1}{2}$ volume and $2\times\frac{1}{4}$ volume of chloroform at about pH 6.5. The traces of chloroform remaining from the extraction were removed under vacuum. An aliquot of a standard solution of 4-C^{14}-aldosterone in ethanol (0.2 ml., 174 dpm) was added to the individual aqueous phases for all the first 24-hour samples before beginning hydrolysis at pH 1.0.

Hydrolysis and extraction at pH 1.0

The urine remaining after the neutral extraction was subjected to hydrolysis at pH 1.0 at room temperature for twenty-four hours by the methods outlined below:

A. A 1000-ml. sample of urine was continuously extracted with 500 ml. of chloroform by the use of a Cohen-type extractor, then further hand-extracted with 3×250 ml. of chloroform. The whole process was repeated for a second twenty-four hours, and finally for a third twenty-four hours. 4-C^{14}-aldosterone was not added again to the second and third 24-hour samples.

B. A 100-ml: sample of urine was allowed to stand with no chloroform present, then extracted by hand with 1×100 ml. of chloroform and 3×25 ml. of chloroform. A further aliquot of the standard 4-C^{14}-aldosterone in ethanol (0.2 ml., 174 dpm) was added to the aqueous phase and the whole process of hydrolysis and extraction was repeated for a second twenty-four hours, and finally (after hydrolysis, extraction and addition of a similar aliquot of 4-C^{14} aldosterone to the aqueous phase) for a third twenty-four hours.

C. A 100-ml. sample of urine was allowed to stand with 100 ml. of chloroform and then extracted by hand with 3×25 ml. of chloroform. The procedure was continued as in Method B.

D. A 100-ml. sample of urine was agitated continuously with 100 ml. of chloroform on a mechanical shaker. The procedure was continued as in Method B.

E. A 100-ml. sample of urine was continuously extracted with 100 ml. of chloroform by the use of a Cohen-type extractor. The procedure was continued as in Method B.

F. A 100-ml. sample of normal urine containing no tritiated aldosterone was extracted at about pH 6.5 with $1\times\frac{1}{2}$ volume and $2\times\frac{1}{4}$ volume of chloroform. An aliquot of 4-C^{14}-aldosterone in ethanol (0.2 ml., 174 dpm) was added to the aqueous phase, which was adjusted to pH 1.0 and extracted immediately with 1×100 ml. and 3×25 ml. of chloroform.

Duplicate aliquots were used in Methods A, B, C, D and F, and triplicate aliquots in Method E. The average room temperature recorded throughout this series of experiments was 22°C. The individual chloroform extracts from an aliquot, and for a particular 24-hour period of hydrolysis, were combined and purified in a preliminary manner

(washing and silica-gel chromatography) by the methods described in detail elsewhere (12). *dl*-Aldosterone (10 μg.) was added to the partially purified extract as a carrier and the mixture chromatographed on a kieselguhr column (30 cm. in length and 1 cm. in diameter, containing 14 Gm. of Celite 545 and 7 ml. of stationary phase). Aliquots of the fractions were spotted on paper and detected, after spraying, by formazan formation and by soda fluoresence (13). The remainders of the fractions were taken to dryness and assayed for C^{14} and H^3 in a liquid scintillator. The semiquantitative estimation of steroid and the C^{14} and H^3 assays agreed for all the relevant fractions, so it was concluded that the H^3 and C^{14} counted was due entirely to aldosterone. The amount of H^3 and C^{14} as aldosterone was therefore taken from the sum of the radioactivity in the appropriate fractions of the Celite column.

RESULTS

The results presented in Table 1 show no significant difference in the yield of tritium as aldosterone, whether the urine was left to stand at pH 1 with or without chloroform or whether it was shaken or extracted continuously with chloroform. This applies to recoveries in the periods 0–24, 24–48 and 48–72 hours. Neither was there any significant difference in the recovery of aldosterone as indicated by the C^{14} assay. The yield of H^3 as aldosterone decreased markedly as the duration of hydrolysis proceeded. The amount produced in the first twenty-four hours was about eight times that in the second twenty-four hours, and one hundred times that in the third twenty-four hours.

. The mean total 72-hour yield of H^3 as aldosterone was 8.4 per cent. This represents a maximal possible yield, since the amount of aldosterone produced by hydrolysis beyond a third 24-hour period would be negligible. The recovery of C^{14}-aldosterone [65 per cent ± 1.4 (S.E.)] following hydrolysis was an indication of the degree of destruction and the efficiency of extraction of the released H^3-aldosterone. The lack of C^{14} in the second and third 24-hour extractions in Method A, where it was not added prior to the hydrolysis except in the first 24-hour period, indicates that these are true values, as there is no indication of carry-over of the C^{14} from the first twenty-four hours. This would otherwise obscure the interpretation of results in Methods B, C, D and E. In Method F, where C^{14}-aldosterone was added to the urine after neutral extraction and the aqueous phase was extracted immediately after being adjusted to pH 1.0, the values for the recovery of C^{14} were 67 and 72 per cent (mean, 70 per cent).

7-H^3-aldosterone (10 μg., 5 μc.) and 4-C^{14}-cortisol (25 μg., 0.5 μc.) were partitioned between water and various organic solvents (10:1, v.v.) which were equilibrated before the experiment. After separation of the phases by centrifugation, the partition coefficients (concentration in solvent/concentration in water) were calculated from the concentrations of tritium and C^{14}. For aldosterone the values were: 33.0 methylene dichloride, 27.2 chloroform, and 3.8 ethyl acetate; the values for cortisol were 10.0, 9.5 and 15.1, respectively.

TABLE 1. PERCENTAGE URINARY RECOVERIES OF RADIOACTIVITY AS ALDOSTERONE AFTER AN INJECTED DOSE OF 7-H^3-*d*-ALDOSTERONE AND AFTER AN ADDED DOSE OF C^{14}-*dl*-ALDOSTERONE

		Methods				
		1000 ml. of urine	100 ml. of urine	100 ml. of urine	100 ml. of urine	100 ml. of urine
		Continuous extraction (A)	Continuous extraction (E)	Continuous shaking (D)	Standing $+CHCl_3$ (C)	Standing; no $CHCl_3$ (B)
First 24 hrs. H^3	a′	7.7	8.3	7.1	7.2	8.2
	b′	8.6	9.1	7.5	7.6	7.1
	c′	—	7.7	—	—	—
Second 24 hrs. (mean)		7.8	8.4	7.3	7.4	7.7
C^{14}	a′	59	66	53	59	68
	b′	74	79	72	78	59
	c′	—	62	—	—	—
Second 24 hrs. H^3	a″	1.3	1.3	0.7	0.9	1.0
	b″	0.4	0.26	0.22	0.33	0.23
	c″	—	0.20	—	—	—
Second 24 hrs. (mean)		0.9	0.9	0.5	0.6	0.6
C^{14}	a″	—	63	63	57	59
	b″	0.8	74	68	65	53
	c″	—	60	—	—	—
Third 24 hrs. H^3	a‴	0.16	0.19	0.05	0.18	0.09
	b‴	0.03	0.03	0.04	0.04	0.003
	c‴	—	—	—	—	—
Third 24 hrs. (mean)		0.1	0.1	0.05	0.11	0.05
C^{14}	a‴	—	70	54	70	50
	b‴	0.9	70	67	67	60
	c‴	—	60	—	—	—
Total 72 hrs. H^3	a	8.5	9.8	7.9	8.3	9.3
	b	9.0	9.4	7.8	8.0	7.3
	c	—	7.9	—	—	—
Total 72 hrs. (mean)		8.8	9.0	7.8	8.2	8.3
Corrected H^3 for recovery from C^{14} counts	a	14.3	14.8	13.7	13.4	15.8
	b	12.2	13.0	11.3	11.4	12.8
	c	—	12.9	—	—	—
Mean		13.3	13.6	12.5	12.4	14.3

DISCUSSION

It might have been expected from the studies of Burstein (14) on the partition coefficients of $C_{21}O_5$ and $C_{21}O_6$ steroids between water and chloroform or ethyl acetate, that ethyl acetate would be the preferred solvent for extraction in the method. However, the partition coefficients obtained in

the present studies, although confirming generally the results of Burstein for cortisol, showed that aldosterone is an exception in this respect and that chloroform is relatively favorable. The same effect is shown in partition by column or paper chromatography when an increase in the amount of ethyl acetate in the mobile phase increases the ratio of the partition coefficients (concentration in water/concentration in solvent) of aldosterone and cortisol [Simpson and Tait (15)]. Chloroform was used therefore in all these studies. On the basis of the partition coefficient obtained, methylene chloride would have yielded similar results.

There is general agreement that extraction of the urine at pH 1.0 for twenty-four hours with chloroform, whatever the method, is more efficient than immediate extraction at pH 1.0 or incubation of the urine with β-glucuronidase (6, 9), for the release of aldosterone.

The results of the experiments reported here indicate that there is very little difference in the yield of aldosterone, whether the urine is left at pH 1.0 for twenty-four hours with or without chloroform and without agitation, or whether it is shaken with chloroform or continuously extracted. This is in general agreement with the conclusions of Mattox and Lewbatr (8) and Kliman and Peterson (10).

Kliman and Peterson (10) have shown that from urine adjusted to pH 2.0, a second 24-hour hydrolysis will yield 50 per cent more aldosterone than that produced in the first 24-hour period. The same workers (10) allowed the urine to stand at pH 1.0 with no organic solvent present, and found that a second 24-hour period of hydrolysis produced about 14 per cent of the aldosterone released in the first period, and that a third 24-hour period produced less than 3 per cent as much. Dyrenfurth and Venning (5) and Sobel *et al.* (9) found a larger proportion released in the second twenty-four hours under these conditions. The data of Kliman and Peterson at pH 1.0 agree closely with the results of the experiments reported here, which are independent of the method of hydrolysis used. However, the data in Table 1 suggest that the yield in a second 24-hour period of hydrolysis can be, for undetermined reasons, rather variable. The recovery of C^{14}-aldosterone from urine not left at pH 1.0 for twenty-four hours but extracted immediately and otherwise subjected to the same purification procedure, was 67 and 72 per cent (mean, 70 per cent), whereas the recovery from urines extracted for twenty-four hours was 65 ± 1.4 per cent S.E. This suggests only a slight destruction of aldosterone under these conditions. However a 5 per cent destruction under these conditions would, as Kliman and Peterson (10) have indicated, make continuous hydrolysis for forty-eight hours produce no more aldosterone than during twenty-four hours. Even if the solvent were changed at frequent intervals or replaced at the intermediate 24-hour stage, the yield would be only about 10 per cent greater. The method of continuous extraction with a Cohen-type extractor is actually a

method of continuous mixing without fresh solvent being introduced. However, even if a true continuous extraction method were used, it is doubtful whether a 48-hour period would be more advantageous except for the most demanding requirements.

On the basis of these results on the efficiency of hydrolysis and the degree of destruction, pH 1.0 appears to be the most appropriate H^+ ion concentration to use, especially since it has been shown (10) that at both higher and lower values the amount of recoverable aldosterone in twenty-four hours is less than at pH 1.0. At pH 0.5, the rate of aldosterone release from the 3-oxo conjugate is faster than at pH 1.0 (10), but there is increased destruction due to the acid. Although this could possibly be considerably reduced by continuous extraction and the use of fresh solvent at frequent intervals, in routine laboratory procedures the accurate measurement of pH values lower than 1.0 would be difficult.

The total amount of aldosterone released (corrected for C^{14} recovery) will furnish an estimate of the amount of conjugate, assuming that the free aldosterone is just as sensitive as the steroid moiety in the conjugate to acid conditions. The mean total amount of conjugate, in the series of experiments reported here, was estimated in this way to be 13.2 per cent. The urine in this study was pooled from 4 female patients and hence this value is fairly representative for normal women. Also the percentage of aldosterone released in the first twenty-four hours corresponds with the mean values for 5 normal subjects as reported by Flood *et al.* (13) and for 6 normal subjects as reported by Jones *et al.* (4). This is to be contrasted with a total glucuronide excretion of 51 per cent in normal subjects (13).

The percentage yield of H^3 in the first twenty-four hours is almost half that reported by Kliman and Peterson (10), whose data on the characteristics of acid hydrolysis conditions otherwise agree with ours. The main difference is that Kliman and Peterson (10) injected tritiated *dl*-aldosterone, whereas in the study reported here the injected material was tritiated *d*-aldosterone. This is additional evidence suggesting that the *l*-enantiomorph is not metabolized to the 3-oxo conjugate, as previously suggested by Tait (16) to explain the difference in the values of secretion rates as calculated by the two groups. Recently Ulick (17) obtained more conclusive evidence after injecting a mixture of 4-C^{14}-*d*-aldosterone and H^3-*dl*-aldosterone and also nonradioactive *dl*-aldosterone and 7-H^3-*d*-aldosterone; he found that the *l*-form is metabolized neither to the tetrahydroaldosterone glucuronide nor to the conjugate referred to in this study.

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THE PROTEIN ANABOLIC ACTION OF MESTANOLONE

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ABSTRACT

A controlled metabolic study of the steroid mestanolone was conducted on 20 females with chronic pulmonary tuberculosis, of whom 5 served as controls and 15 received 100 mg. of mestanolone daily. A statistically significant increase in weight occurred in the treated patients and was associated with a significant retention of nitrogen, potassium and phosphorus in the ratio in which they are normally found in protoplasm. The mean gain in body weight resulting from two courses of mestanolone (eighteen days each with an interval of eighteen days between) was 2.10 Kg., and the mean nitrogen retention was 1.89 Gm. daily.

THE protein anabolic action of testosterone and its derivatives is well known, and the vast body of literature which has accrued has been ably and critically collated by Dorfman and Shipley (1).

Unfortunately the most powerful anabolic steroids have androgenic effects which occur with sufficient frequency to limit their clinical usefulness. It is logical, therefore, to investigate modified androgens to see whether the anabolic and virilizing actions can be dissociated.

The steroid mestanolone (17-methyl-androstan-17-ol-3-one) seemed to be suitable for investigation as it was reputed to be of low androgenic potency while retaining anabolic activity.

In the castrated rat it was found that mestanolone had about 75 per cent of the effect of testosterone in promoting nitrogen retention and weight gain (2). Clinically, weight gain has been recorded in uncontrolled studies (3–5). In one small metabolic study, an increase in weight and in nitrogen retention was noted; unfortunately only an abstract of the paper was published so that no figures are available (6). The virtual absence of virilizing effects has been reported in the human female (3, 7, 8).

MATERIAL AND METHODS

Twenty women suffering from chronic pulmonary tuberculosis were studied. Fifteen received treatment with mestanolone (50 mg. sublingually twice daily) and 5 received placebo tablets. The tuberculosis had not been responding to prolonged chemotherapy,

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and the patients were all underweight for height and age, and had lost weight during the previous four weeks.

A standard diet (based on a six-day "block") was given throughout. This averaged 2600 calories daily, containing 90 Gm. of protein, 14.1 Gm. of nitrogen, 84 mEq. of potassium, and 1.638 Gm. of phosphorus.

Urine was collected as 24-hour specimens, and feces as six-day specimens with the use of a carmine marker. Body weight was recorded (naked, before breakfast and after voiding) on every sixth day.

The study was divided into five periods, each of eighteen days' duration. The first was a control period for each patient; periods 2 and 4 were the treatment periods; and periods 3 and 5 were the recovery periods.

Urinary and fecal potassium were measured by flame photometry (9). Urinary and fecal nitrogen were measured by a Kjeldal method (10). Urinary and fecal phosphorus were measured by the method of Fiske and SubbaRow (11).

RESULTS

Body weight

Table 1 shows the body weight of each patient at the end of each period and the gains during the two treatment periods. In the control group there was little change throughout the whole study. In the mestanolone-treated

TABLE 1. BODY WEIGHT AT THE END OF EACH 18-DAY PERIOD AND THE GAIN DURING TREATMENT PERIODS 2 AND 4

Patients	Age (yrs.)	Height (cm.)	Body weight (Kg.)						
			At end of period 1	At end of period 2	Gain in period 2	At end of period 3	At end of period 4	Gain in period 4	At end of period 5
Treated with Mestanolone									
P.S.	21	154.5	45.36	46.10	0.74	46.07	46.92	0.85	46.89
E.G.	23	156	49.50	50.41	0.91	50.41	51.26	0.85	51.26
M.N.	27	152.5	48.68	49.67	0.99	49.61	50.23	0.62	50.17
E.B.	31	154.5	43.55	44.77	1.22	44.77	46.30	1.53	46.30
E.P.	34	154	41.22	42.19	0.97	42.19	43.50	1.31	43.47
S.W.	29	166.5	56.19	57.16	0.97	57.13	58.10	0.97	58.10
P.T.	30	162.5	53.96	55.18	1.22	55.12	55.97	0.85	55.94
N.A.	39	159	51.77	52.91	1.14	52.82	54.38	1.54	54.30
R.G.	25	150	37.65	38.73	1.08	38.73	39.61	0.88	39.61
S.B.	27	151	45.39	46.75	1.36	46.69	48.34	1.65	48.28
G.H.	32	155	53.44	54.06	0.62	54.00	55.05	1.05	55.05
I.W.	21	166	53.44	54.09	0.65	54.00	54.82	0.82	54.79
K.G.	22	150	37.76	38.36	0.60	38.36	39.50	1.14	39.50
R.S.	29	154.5	33.09	33.89	0.80	33.89	35.31	1.42	35.28
N.S.	22	164	47.66	48.63	0.97	48.60	49.57	0.97	49.51
			Mean gain		*0.99*			*1.11*	*2.10*
Controls									
J.G.	21	157.5	46.78	46.75	−0.03	46.78	46.78	0	46.72
P.Y.	38	155	53.53	53.56	0.03	53.50	53.50	0	53.53
I.F.	24	160	49.47	49.47	0	49.50	49.50	0	49.47
A.P.	31	154	46.27	46.30	0.03	46.27	46.27	0	46.30
B.Q.	25	166.5	54.89	54.80	−0.09	54.77	54.71	−0.06	54.80
			Mean gain		*−0.01*			*−0.01*	*−0.02*

TABLE 2. RETENTION OF NITROGEN DURING EACH 18-DAY PERIOD

Patients	Retention of nitrogen (Gm./24 hrs.)				
	Period 1	Period 2	Period 3	Period 4	Period 5
Treated with Mestanolone					
P.S.	−0.17	1.36	−0.08	1.22	−0.10
E.G.	−0.20	1.49	0.11	1.69	0.01
M.N.	−0.13	1.95	−0.01	1.88	−0.03
E.B.	−0.37	2.45	0.10	2.77	0.10
E.P.	−0.10	1.61	0.03	1.87	−0.12
S.W.	−0.20	2.05	−0.07	1.97	0.01
P.T.	−0.18	2.06	−0.03	1.54	−0.02
N.A.	−0.22	1.83	−0.01	1.83	−0.21
R.G.	−0.20	2.24	0.03	2.33	0.08
S.B.	−0.08	3.09	−0.07	2.80	−0.01
G.H.	−0.23	1.46	−0.06	1.32	0.06
I.W.	−0.07	1.12	−0.08	1.53	−0.01
K.C.	−0.08	1.25	0.12	1.93	0.11
R.S.	−0.33	1.39	0.22	1.93	−0.07
N.S.	−0.35	1.64	−0.08	2.11	−0.03
Mean	*−0.19*	*1.80*	*0.01*	*2.01*	*−0.02*
Controls					
J.C.	−0.07	0.03	−0.03	−0.03	−0.01
P.Y.	−0.08	−0.22	0.01	−0.04	0.08
I.F.	−0.12	0.01	−0.08	0.07	−0.10
A.P.	−0.35	−0.10	−0.11	−0.03	−0.07
B.Q.	−0.02	−0.03	−0.03	−0.02	−0.01
Mean	*−0.16*	*−0.07*	*−0.05*	*−0.01*	*−0.02*

group there was a mean gain of 0.99 Kg. during period 2 and a mean gain of 1.11 Kg. during period 4. The control patients showed a mean *loss* of 0.01 Kg. during periods 2 and 4. Statistically the difference between these means was highly significant ($P < 0.001$).

During the recovery periods body weight remained stationary or there were slight losses of up to 0.09 Kg. in the treated patients.

Nitrogen balance

The nitrogen retention in grams per diem is shown in Table 2. In each case the retention was calculated with reference to the mean of the preceding control or recovery period.

In the control period all patients were in a state of negative nitrogen balance. In period 2 the mean 24-hour retention in the treated group was 1.80 Gm., whereas in the controls the nitrogen value was −0.07. In period 4 the mean 24-hour nitrogen retention in the treated group was 2.01 Gm., whereas in the control group the value was −0.01 Gm. In the recovery periods the treated group remained more or less in equilibrium.

The difference between the means of the treated and control groups was highly significant in both period 2 and period 4 ($P < 0.001$).

Potassium balance

This followed a pattern similar to that for nitrogen, with a negative balance during the control period, positive and negative fluctuations in the recovery periods, and definite retention during periods 2 and 4 in the treated patients. The potassium retention in each patient is shown in Table 3. In period 2 the mean 24-hour retention for the treated patients was 4.87 mEq., and for the control group it was 0.10 mEq. In period 4 the mean 24-hour potassium value was 5.45 mEq. in the treated group, and −0.07 mEq. in the control group. Statistically the difference between these means was highly significant ($P < 0.001$).

Phosphorus balance

This also followed much the same pattern as that for nitrogen balance, with slightly fluctuating equilibrium during periods 1, 3 and 5 and definite retention during periods 2 and 4. Phosphorus retention in each patient is shown in Table 4. In period 2 the treated group showed a mean 24-hour phosphorus retention of 113.5 mg. and the control group a mean of 3 mg. In period 4 the treated group showed a mean 24-hour retention of 130 mg., and the control group a mean of 0. The difference between these means was statistically highly significant ($P < 0.001$).

TABLE 3. RETENTION OF POTASSIUM DURING EACH 18-DAY PERIOD

Patients	Retention of potassium (mEq./24 hrs.)				
	Period 1	Period 2	Period 3	Period 4	Period 5
			Treated with Mestanolone		
P.S.	−0.50	3.72	1.00	4.11	−0.58
E.G.	−0.50	4.36	−0.83	4.58	0.10
M.N.	−0.33	5.28	−0.67	5.11	−0.08
E.B.	−0.33	6.97	0.17	7.72	0.50
E.P.	−0.67	4.39	0.50	5.00	−0.17
S.W.	−0.83	5.47	−0.25	4.92	0.33
P.T.	−0.83	5.53	0.00	4.17	−0.67
N.A.	−0.17	5.11	0.92	5.03	−0.25
R.G.	−0.67	6.08	−0.17	6.22	0.10
S.B.	−0.17	8.16	0.25	10.19	−0.67
G.H.	−0.67	4.14	0.10	3.94	0.58
I.W.	−0.08	3.28	−0.83	4.45	−0.19
K.C.	−0.17	3.06	−0.42	4.86	0.33
R.S.	−0.17	3.58	0.10	6.22	−0.25
N.S.	−0.33	3.97	−0.50	5.22	−0.08
Mean	*−0.43*	*4.87*	*0.00*	*5.45*	*−0.07*
			Controls		
J.C.	−0.33	0.19	−0.08	0.08	−0.08
P.Y.	−0.17	−0.29	−0.17	−0.31	0.10
I.F.	−0.50	0.10	−0.03	−0.03	−0.03
A.P.	−0.17	0.39	−0.10	0.11	−0.17
B.Q.	−0.17	0.11	0.00	−0.11	−0.33
Mean	*−0.27*	*0.10*	*−0.08*	*−0.07*	*−0.08*

TABLE 4. RETENTION OF PHOSPHORUS DURING EACH 18-DAY PERIOD

Patients	Retention of phosphorus (mg./24 hrs.)				
	Period 1	Period 2	Period 3	Period 4	Period 5
Treated with Mestanolone					
P.S.	− 2	90	10	109	6
E.G.	−10	95	−10	109	− 5
M.N.	8	127	− 7	123	−10
E.B.	− 4	156	13	177	1
E.P.	7	106	12	124	6
S.W.	7	124	1	115	1
P.T.	−18	129	1	96	5
N.A.	−10	115	− 6	120	−11
R.G.	−10	137	4	146	0
S.B.	−12	102	−13	238	0
G.H.	−13	95	11	91	1
I.W.	1	64	−28	93	0
K.C.	1	77	1	117	15
R.S.	0	95	6	158	−10
N.S.	0	100	− 8	134	0
Mean	*− 4*	*113.5*	*− 1*	*130*	*0*
Controls					
J.C.	3	2	− 8	1	0
P.Y.	− 3	9	− 1	−2	− 2
I.F.	− 2	−1	− 1	2	− 1
A.P.	4	3	6	−1	− 5
B.Q.	− 7	2	− 3	0	1
Mean	*− 1*	*3*	*− 1.5*	*0*	*− 1.5*

Side-effects

In this study, in which mestanolone was given daily for two periods of eighteen days each with eighteen days between, side-effects were minimal. Twelve of the 15 treated patients noted an increased appetite and 2 noted a change in menstrual pattern, *i.e.*, a delay of menses by one week while taking the drug. No patient manifested hirsutism or hoarseness.

DISCUSSION

The results demonstrate that in this group of women with chronic pulmonary tuberculosis, mestanolone produced a highly significant gain in body weight accompanied by retention of nitrogen, potassium and phosphorus.

If the retained nitrogen, potassium and phosphorus are fully utilized in the formation of new protoplasm then the theoretical amount of nitrogen, based on the values for potassium or phosphorus, can be calculated from their normal proportions in that tissue. Table 5 shows the results of these calculations for periods 2 and 4, with the use of the data given in Tables 2, 3 and 4. The theoretical value for nitrogen based on potassium is given by the formula "retained N(Gm.) = retained K(mEq.)/2.7"; and the theo-

TABLE 5. COMPARISON BETWEEN MEASURED NITROGEN RETENTION AND THEORETICAL NITROGEN RETENTION BASED ON POTASSIUM RETENTION (N(Gm.) = K(mEq.)/217) AND THEORETICAL NITROGEN RETENTION BASED ON PHOSPHORUS RETENTION (N(Gm.) = P(mg.) × 14.7/1000)

Patients	Period 2			Period 4		
	Measured nitrogen retention	Theoretical N based on K	Theoretical N based on P	Measured nitrogen retention	Theoretical N based on K	Theoretical N based on P
Treated with Mestanolone						
P.S.	1.36	1.38	1.32	1.22	1.52	1.50
E.G.	1.49	1.61	1.40	1.69	1.70	1.50
M.N.	1.95	1.95	1.87	1.88	1.89	1.18
E.B.	2.45	2.79	2.29	2.77	2.86	2.59
E.P.	1.61	1.62	1.56	1.81	1.85	1.82
S.W.	2.05	2.02	1.82	1.79	1.82	1.69
P.T.	2.06	2.05	1.90	1.54	1.54	1.41
N.A.	1.83	1.89	1.69	1.83	1.90	1.76
R.G.	2.24	2.25	2.01	2.33	2.46	2.15
S.B.	3.09	2.98	2.82	3.80	3.77	3.50
G.H.	1.46	1.53	1.40	1.32	1.47	1.34
I.W.	1.12	1.22	0.94	1.53	1.68	1.37
K.G.	1.25	1.12	1.13	1.93	1.80	1.72
R.S.	1.39	1.32	1.40	1.93	2.30	2.32
N.S.	1.64	1.47	1.47	2.11	1.94	1.97
Controls						
J.G.	0.03	0.07	0.03	−0.01	0.03	0.02
P.Y.	0.22	0.33	0.13	−0.04	−0.11	−0.03
I.F.	0.01	0.02	−0.02	0.17	0	0.03
A.P.	0.11	0.14	0.04	−0.03	0.04	−0.02
B.Q.	0.03	0.04	0.03	−0.02	0.04	0

retical value for nitrogen based on phosphorus is given by the formula "retained N(Gm.) = retained (P(mg.) × 14.7/1000," as suggested by Reifenstein, Albright and Wells (12).

The close correspondence of these theoretical values with the observed values supports the view that the retained nitrogen, potassium and phosphorus were in fact used in making new protoplasm. Any marked divergence would have suggested the involvement of other factors; thus, the weight gain might have been due to fluid retention, or calcium retention might have accounted for a considerable proportion of the phosphorus. The data shown in Table 5 suggest that neither of these factors was of importance.

Although not as potent as testosterone, mestanolone does not compare to disadvantage in its anabolic power. For example, a nitrogen retention of 2–4 Gm. daily has been reported during administration of testosterone in a dosage of 90–100 mg. daily (13, 14), and a retention of 1.0–1.5 Gm. during administration of 100 mg. three times a week (15). In the present study the range of nitrogen retention was 1.12–3.80 Gm. (mean, 1.89 Gm.) daily during treatment with mestanolone, 100 mg. daily.

Owing to the small number of patients involved and the short period of

reatment, no definite statement can be made about possible virilization ther than that mestanolone is not highly virilizing.

It is concluded that mestanolone is a moderately potent protein anabolic gent suitable for clinical use.

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THE DISPOSAL OF PLASMA 17-HYDROXY-CORTICOSTEROIDS. I. EXPONENTIAL DISPOSAL FROM A SINGLE COMPARTMENT*

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ABSTRACT

Many investigators have stated or implied that the distribution and disposal of plasma 17-hydroxycorticosteroids (17-OH-CS) can be approximated by assuming that these substances are distributed in the body as though they were in a single compartment and are disposed of at a rate proportional to concentration. In the present investigation, cortisol was given by intravenous infusion and the changes in plasma 17-OH-CS concentrations were examined. The data obtained were then compared with the predictions of the single compartment-exponential disposal theory. The concentration of plasma 17-OH-CS was studied in 4 experimental situations: 1) after cessation of constant intravenous infusions of cortisol at slow rates; 2) after administration of large amounts of cortisol; 3) after equilibration attained during constant infusions of cortisol; and 4) during the early period of infusions of cortisol. In all 4 experimental situations, there was a poor correspondence between the predicted and the actual data. It is concluded that 17-OH-CS do not disappear from the plasma as though they were distributed in a single compartment and disposed of at a rate proportional to concentration. Since plasma 17-OH-CS disposal does not follow first-order kinetics, the half-time of plasma 17-OH-CS is not independent of concentration. Methods for quantitation of adrenocortical function that utilize the assumptions of single-compartment distribution and exponential disposal of plasma 17-OH-CS are based on a theory which does not describe the data accurately.

THERE are many facts which suggest that a mathematical description of plasma 17-hydroxycorticosteroid (17-OH-CS) distribution and disposal would be complicated. Among these facts are the number of 17,21-dihydroxy-20-ketosteroids detectable as Porter-Silber chromogens (1), the limitation of diffusion into tissues by plasma protein binding of 17-OH-CS (2–4), the known differences in the apparent volume of distribu-

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tion and the rate of disposal of cortisol when determined after tracer and load doses of cortisol (5–7), and the time necessary to insure equilibration of 17-OH-CS within the body fluids (4, 5). In addition, it has been postulated that protein-bound 17-OH-CS would not be available for direct metabolism by the usual routes and that this might account for the delay in the disposal of these substances when plasma protein binding of 17-OH-CS is increased (2, 3, 4, 8). However, the fact that cortisol is the preponderant 17-OH-CS in human plasma (6), the possibility that there is a single rate-limiting step in the degradation of cortisol (9), and the seemingly satisfactory agreement of plasma 17-OH-CS disposal data with first-order kinetics when the logarithm of concentration is plotted as a function of time (5, 7) all suggested that a simple mathematical approximation could be made. These observations led us and other investigators to assume, as an approximation in calculating disposal rate data, that plasma 17-OH-CS are distributed in the body as though they were in a single compartment and are disposed of at a rate proportional to concentration (1, 4, 5, 10–14). The purpose of the present investigation was to examine how closely the actual changes in the concentration of plasma 17-OH-CS after intravenous infusions of cortisol correspond with the changes predicted by the single compartment-exponential disposal theory.

METHODS

The methods for the collection of blood specimens, the determination of plasma 17-OH-CS and the administration of constant infusions of cortisol have been described elsewhere (15, 16). Dexamethasone (0.5 mg. every six hours or 1.0 mg. every eight hours) was given by mouth during all of the infusions, to suppress endogenous 17-OH-CS production (17, 18). When plasma 17-OH-CS concentrations were studied during the initial part of a cortisol infusion, dexamethasone administration was started between 11:00 and 12:00 p.m. on the night preceding the infusion. Cortisol infusions were started between 7:00 and 9:00 a.m. The subjects were fasting. The cortisol was dissolved in 5 per cent dextrose in water and all infusions were given intravenously at a rate of 40 to 60 ml. per hour. Plasma 17-OH-CS levels were determined in duplicate and the mean values were recorded except where noted. A mechanical device[1] that determines tangents to curves accurately and rapidly was used to measure the rate of change of concentration of plasma 17-OH-CS with respect to time on free-hand curves drawn through the data (19).

RESULTS

Suppression of endogenous plasma 17-OH-CS production with dexamethasone

Seven normal male and 2 normal female subjects were given 1 mg. of dexamethasone by mouth at midnight, and 0.5 mg. at 8:15 a.m. In plasma from blood specimens obtained at 8:00 a.m. and 2:00 p.m., the mean 17-OH-CS concentrations were 16 μg. and 2 μg. per liter, with ranges of

[1] Gerber Derivimeter, Gerber Scientific Instrument Company, Hartford, Connecticut.

0–58 μg. and 0–18 μg. per liter, respectively. Plasma 17-OH-CS concentration in untreated normal subjects averages 160 μg. and 103 μg. per liter at 8:00 a.m. and 2:00 p.m. respectively (20). Although administration of dexamethasone suppressed endogenous 17-OH-CS production in every subject, suppression was incomplete in a few, particularly at 8:00 a.m.

Plasma 17-OH-CS concentrations after cessation of constant infusions of cortisol at slow rates

Seven subjects without known hepatic or endocrine disorders were given constant intravenous infusions of cortisol at rates not exceeding 165 μg. per Kg. of body weight per hour for six hours (Subjects 2 to 8 in Table 1). The infusions were then discontinued and blood specimens were obtained at intervals during the subsequent two hours. One patient with panhypopituitarism was studied in the same way (Subject 1 in Table 1). The data are shown in Table 1 and Figure 1. Duplicate plasma 17-OH-CS determinations were not performed in this portion of the study.

Plasma 17-OH-CS concentrations after cessation of infusions of cortisol at rapid rates

Three normal subjects were given constant intravenous infusions of cortisol at a rate of 440 μg. per Kg. of body weight per hour for six to eight hours (Subjects 1, 2 and 3 in Table 2). The infusions were then discontinued and blood specimens were obtained at intervals over the subsequent eight hours. Two additional normal subjects were given intravenous infusions of 1 mg. of cortisol per Kg. of body weight over three-minute periods and blood specimens were obtained during the subsequent six hours (Subjects 4 and 5 in Table 2). The data are shown in Table 2 and Figure 2.

Plasma 17-OH-CS concentrations six hours after starting constant infusions of cortisol

Six normal subjects were given intravenous infusions of cortisol at rates of 55, 110 and 220 μg. per Kg. of body weight per hour for six hours on successive days. The order of the infusion rates was reversed in 3 of the subjects. Blood specimens were obtained five and six hours after the start of the infusions. In Table 3 and Figure 3 are recorded the plasma 17-OH-CS concentrations at six hours. In 2 instances, one of the duplicate six-hour plasma samples was lost. In these cases, the mean of the five and six-hour values was used. It was assumed that equilibration of plasma 17-OH-CS concentration was approximated at the end of these six-hour infusions. The mean of the six-hour plasma 17-OH-CS concentrations after the 55 and 110 μg. per Kg.-hour infusions was within 30 μg. per liter of the mean of the five-hour concentrations. In 2 subjects, the 220 μg. per Kg.-hour infusions

TABLE 1. PLASMA 17-HYDROXYCORTICOSTEROID CONCENTRATIONS AFTER CESSATION OF SIX-HOUR INTRAVENOUS INFUSIONS OF CORTISOL AT SLOW RATES

Subjects				Rate of cortisol infusion (μg./Kg.-hr.)	Plasma 17-OH-CS (μg./L.) after cessation of cortisol infusion							
					Time in hours							
No.	Sex & age (yrs.)	Ht. (cm.)	Wt. (Kg.)		0	¼	½	¾	1	1½	2	Slope (k in hrs.$^{-1}$)
1	M 31	150	31	50	324	262	216	210	184	158	140	0.92
2	F 67	162	70	75	320		280		232	202	186	1.00
3	F 23	168	68	75	312		234		206	196	164	1.32
4	M 29	180	73	165	424		318		250	220	200	1.36
5	M 30	180	82	110	300		215		150	110	90	1.18
6	M 29	180	79	110	380		292		220	180	176	1.13
7	F 27	170	64	110	372		246		256	188	176	—
8	M 74	165	52	110	434		324		352	280	220	—
												Mean = 1.15

TABLE 2. PLASMA 17-HYDROXYCORTICOSTEROID CONCENTRATIONS AFER CESSATION OF INTRAVENOUS INFUSIONS OF CORTISOL AT RAPID RATES

Subjects				Rate of cortisol infusion	Plasma 17-OH-CS (μg./L.) after cessation of cortisol infusion															
					Time in hours															
No.	Sex & age (yrs.)	Ht. (cm.)	Wt. (Kg.)		0	⅛	¼	½	¾	¾	1	1¼	1¾	2	2½	3	4	5	6	7	8
1	M 23	185	90	440 μg./Kg.-hr. for 8 hrs.	1087		774		699		706	657	496	507	436	337	268	206	182	142	116
1	M 24	180	72	440 μg./Kg.-hr. for 8 hrs.	780		532		495		443	412	398	344	308	191	184	127	110	89	71
3	M 24	183	91	440 μg./Kg.-hr. for 6 hrs.	1145		804				694			515		351	265	222	178	102	96
4	M 29	180	77	1 mg./Kg for 3 mins.		884	848	764		620	550	570	458	412	352	344	274	193	120		
5	M 23	180	73	1 mg./Kg. for 3 mins.		1206	828	756		676	558	476	441	404	325	286	208	148	89		

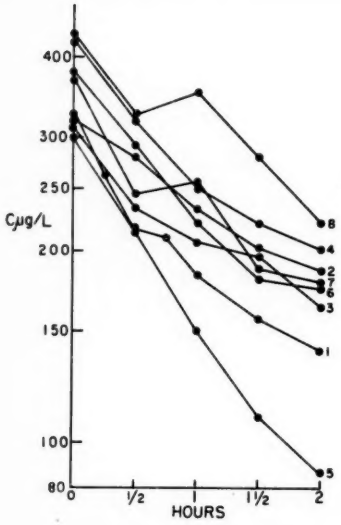

FIG. 1. Plasma 17-OH-CS concentrations (μg./L.) plotted as a function of time in hours after cessation of six-hour intravenous infusions of cortisol at rates not exceeding 165 μg. per Kg.-hour. The numbers at the ends of the lines refer to the subjects in Table 1.

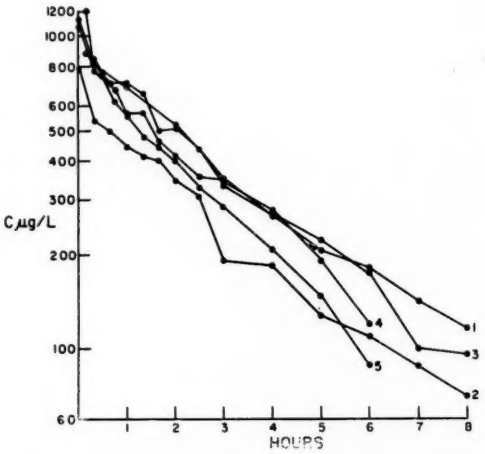

FIG. 2. Plasma 17-OH-CS concentrations (μg./L.) plotted as a function of time in hours after cessation of intravenous injections of large amounts of cortisol. The numbers at the ends of the lines refer to the subjects in Table 2.

TABLE 3. PLASMA 17-HYDROXYCORTICOSTEROID CONCENTRATIONS SIX HOURS AFTER STARTING CONSTANT INTRAVENOUS INFUSIONS OF CORTISOL

Subjects				Plasma 17-OH-CS (μg./L.) cortisol infusion rates (μg./Kg.-hr.)		
No.	Sex & age (yrs.)	Ht. (cm.)	Wt. (Kg.)	55	110	220
1	M24	188	72	237	345	575
2	M25	180	63	228	342	591
3	M24	180	72	192	310	468
4	M23	186	90	235	354	654
5	F21	163	52	230*	340	494
6	F23	165	58	226	280*	439
Mean				*225*	*328*	*537*

* These values represent the mean plasma 17-OH-CS concentrations of single six-hour specimens and duplicate five-hour specimens.

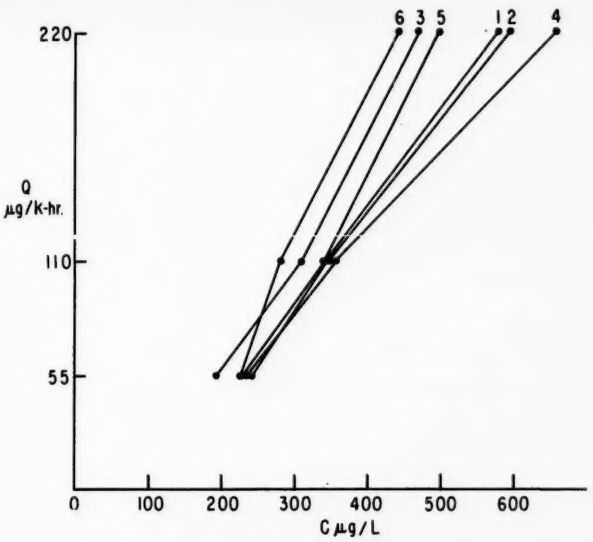

FIG. 3. The rate, Q, of the constant infusion of cortisol (μg. per Kg.-hour) plotted as a function of the equilibrium concentration of plasma 17-OH-CS (μg./L.). The numbers at the top of the lines refer to the subjects in Table 3.

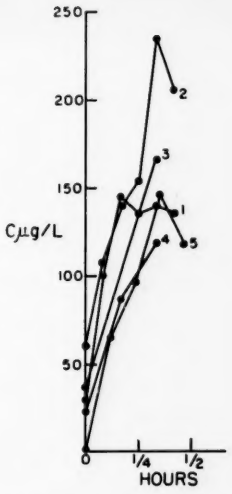

FIG. 4 Plasma 17-OH-CS concentrations (μg./L.) plotted as a function of time in hours after starting intravenous infusions of cortisol at constant rates. The numbers at the ends of the lines refer to the subjects in Table 4.

were continued for an additional two hours, and the mean eight-hour plasma 17-OH-CS concentration was within 30 μg. per liter of the mean six-hour value.

Plasma 17-OH-CS concentrations during the early phase of constant infusions of cortisol

Five normal young males were given intravenous infusions of cortisol at rates of 55 to 110 μg. per Kg. of body weight per hour (Table 4). Blood specimens were obtained at intervals during the first twenty-eight minutes of the infusion. The plasma 17-OH-CS concentrations observed in each subject are shown in Figure 4. The plasma 17-OH-CS levels at the onset of the infusions were higher than expected on the basis of the data on dexamethasone suppression. Perhaps some feature of the experimental situation, possibly anxiety, accounted for the incomplete suppression of endogenous plasma 17-OH-CS production in these studies.

TABLE 4. EARLY PLASMA 17-HYDROXYCORTICOSTEROID CONCENTRATIONS DURING CONSTANT INTRAVENOUS INFUSIONS OF CORTISOL

Subjects				Rate of cortisol infusion (μg./Kg.-hr.)	Slope (μg./L.-hr.)	
No.	Sex & age (yrs.)	Ht. (cm.)	Wt. (Kg.)		Actual	Predicted
1	M24	180	72	110	700	251
2	M23	186	90	110	495	242
3	M29	180	79	75	390	172
4	M27	183	84	55	385	121
5	M24	180	72	55	465	130

DISCUSSION

The single compartment-exponential disposal theory is described by the following equation:

$$\text{(A)} \qquad \frac{dC}{dt} = \frac{Q}{V} - kC$$

where C equals the concentration of plasma 17-OH-CS in μg. per liter, t equals time in hours, Q is the rate of infusion of cortisol in μg. per Kg.-hour, V is the apparent volume of distribution in liters per Kg., and k is a constant in hours^{-1}. Equation (A) will be used to analyze the various experimental situations. The experimental data can then be compared with the theoretical predictions.

Cessation of constant infusions of cortisol at slow rates

With cessation of a constant infusion of cortisol, Q equals zero and the predicted rate of decrease in plasma 17-OH-CS concentration should be described by the following equation:

$$\text{(B)} \qquad -\frac{dC}{dt} = kC.$$

That is, dC/dt when plotted as a function of C should be a straight line passing through the origin of the axes. The data obtained after cessation of six-hour cortisol infusions at slow rates (Table 1) were plotted (concentration as a function of time), and smooth lines were drawn through the points. In 2 cases (Subjects 7 and 8), it was not possible to select a single smooth curve to describe the data, and these 2 cases were omitted from the analysis. The dC/dt was determined at varying concentrations and the results obtained were plotted in Figure 5. As predicted, there was a linear relationship between dC/dt and C over the investigated range of concentrations; that is, the data approximated straight lines. However, in contrast

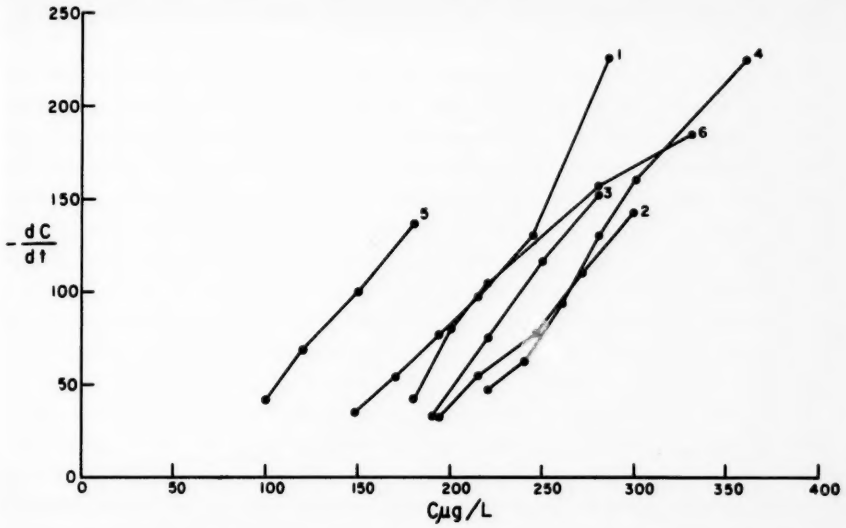

FIG. 5. The rate of change of plasma 17-OH-CS concentration with respect to time after cessation of a constant infusion of cortisol at a slow rate, plotted as a function of plasma 17-OH-CS concentration. The numbers at the top of the lines refer to the subjects in Table 1.

to the theoretical prediction, in no case in Figure 5 would straight projections from the lines go through the origin of the axes.

There is an alternative and more commonly utilized method for analysis of these data. The single compartment-exponential disposal theory postulates that disposal of cortisol follows first-order kinetics. The correspondence of the data with first-order processes can be studied by examining the change in the logarithm of concentration as a function of time. The integrated form of equation (B) is:

(C) $$\log C = -0.43\,kt + \log C_o$$

where C_o equals C when t equals zero. According to equation (C), a plot of log C as a function of time should give a straight line intersecting the Y axis at log C_o. When the data in Table 1 were plotted in this fashion (Fig. 1), the lines, in general, were concave. The difference between some of the data lines in Figure 1 and straight lines is not great, and this has led some investigators to conclude that the disposal of plasma 17-OH-CS is adequately described by the single compartment-exponential disposal theory. However, when the data were plotted to show the change in concentration with respect to time as a function of concentration (Fig. 5), the significance

of the discrepancy between the theory and the data is more apparent (19, 21).

Objections might be raised to the abstract nature of differential equation (B), the drawing of free-hand curves and the use of a mechanical tangent-measuring device in the preparation of the data shown in Figure 5. The objections to this approach on the grounds of abstractness can be countered by the fact that the rationale for the use of the semilogarithmic plot is based on equation (C), the integral of equation (B), and surely a no less abstract expression of the theory. Furthermore, although alternative free-hand curves can be drawn through the data of Table 1 which produce slightly different lines than those of Figure 5, the results persistently do not conform to the predictions of equation (B). The tangent-measuring device has previously been shown to give reproducible and accurate results (19).

All of the studies after the constant infusions of cortisol at slow rates were completed within two hours after cessation of the infusions. Conceivably, the rate of fall of the concentration of plasma 17-OH-CS during these two-hour periods might be greatly influenced by delays in mixing (4, 5). Observations over a longer period of time might reveal a closer correspondence between the theoretical predictions and the data.

Cessation of infusions of large amounts of cortisol

. These studies were continued over six-hour or eight-hour periods after cessation of the infusions of cortisol. In Figure 2, the data are plotted to show the relation of the logarithm of the concentration of plasma 17-OH-CS to time. According to the disposal theory as stated in equation (C), the data should form straight lines. During the first hour, for every subject, the data formed curves that were concave upwards in contrast to the theoretical predictions. After the first hour, the data for the individual subjects might be described as roughly conforming to straight lines. However, semilogarithmic plots can easily obscure significant deviations from exponential processes (19).

In Figure 6 the values for dC/dt derived from Table 2 are plotted as functions of concentration. The lines in Figure 6 are not simple straight lines passing through the origin of the axes, in contrast to the theoretical predictions.

Equilibration during constant infusion of cortisol

After equilibration is achieved during the constant infusion of cortisol, $dC/dt = 0$ and equation (A) would predict that:

(D) $$Q = kVC_{eq}$$

that is, the theory predicts that the rate of cortisol infusion, Q, is propor-

tional to C_{eq}, the equilibrium concentration of plasma 17-OH-CS. The product of the disposal rate constant, k, and the apparent volume of distribution, V, form the proportionality constant. However, in contrast to the theoretical predictions, when the means of the values of C_{eq} in Table 3 are substituted in equation (D), kV is not constant with change in Q. For a Q equal to 55, 110 and 220, kV is found to equal 0.244, 0.335 and 0.410, respectively. Manipulation of the data reveals that Q is proportional to C_{eq}

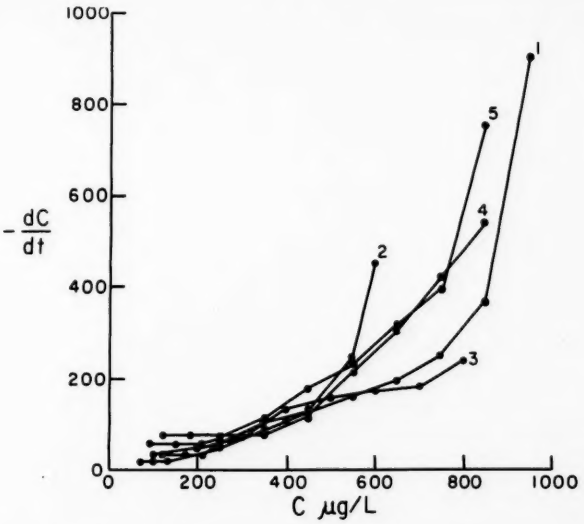

FIG. 6. The rate of change of plasma 17-OH-CS concentration with respect to time after intravenous injection of a large amount of cortisol, plotted as a function of plasma 17-OH-CS concentration. The numbers at the ends of the lines refer to the subjects in Table 2.

plus a constant term of −64. This result can be visualized in Figure 3. Equation (D) predicts that the data will fall on a straight line which, when projected, should extend through the origin of the axes. The data on the individual subjects in Figure 3 do form fairly straight lines but in no instance are these lines directed toward the origin of the axes. These results are at variance with the predictions of the theory.

It is to be emphasized that these studies on the relation of the rate of cortisol infusion to the equilibrium concentration of plasma 17-OH-CS were performed at a time when mixing must be fairly complete. Therefore, in this equilibration portion of the study, the discrepancy between the predictions of the single compartment-exponential disposal theory and the data are not attributable to delayed mixing.

Plasma 17-OH-CS concentrations during the early phase of constant infusions of cortisol

To estimate the rate of change of concentration of plasma 17-OH-CS with respect to time during the early phase of constant cortisol infusions, straight lines were drawn through the initial and 10-minute concentrations in each case in Figure 4 and the slopes of the lines were measured. These values are recorded as actual slopes in Table 4.

An estimate can be made of the expected rate of change of plasma 17-OH-CS concentration (predicted slopes) early in a constant cortisol infusion by utilizing the single compartment-exponential disposal theory and neglecting delays due to mixing time. The apparent volume of distribution of plasma 17-OH-CS after intravenous injection of 1 mg. of cortisol per Kg. of body weight is 0.9 liter per Kg. (5). After an intravenous tracer dose of C^{14}-labeled cortisol, the compound is distributed over an apparent volume of 0.21 to 0.45 liter per Kg. (5). In terms of the quantity of cortisol administered, the infusions of cortisol recorded in Table 4 might be regarded as intermediate between the tracer and the 1 mg.-per-Kg. load of cortisol. As an estimate, let V in equation (A) equal 0.3 liter per Kg. and let k equal 1.15 hour^{-1}, the mean value from Table 1. Substituting the plasma 17-OH-CS concentrations at five minutes from Figure 4 into equation (A) will give the predicted dC/dt, which can be compared with the actual slopes recorded in Table 4. For example, in the case of Subject 1—at five minutes, C equals 100 μg. per liter and it would be predicted that:

$$\frac{dC}{dt} = (-1.15)(100) + \frac{110}{0.3} = 251.$$

The data in the other subjects were similarly analyzed. The results are recorded in Table 4 where it can be seen that the actual slopes ranged from 385 μg. to 700 μg. per liter-hour, whereas the predicted slopes ranged from 121 μg. to 251 μg. per liter-hour. Had a larger value been selected for V in the calculation of the slope, the discrepancy between the actual and the predicted data would have been even greater.

The results of the comparison between the predictions of the single compartment-exponential disposal theory and those of the data in the foregoing 4 experimental situations leads to the conclusion that the theory does not closely fit the data. Sheuer and Bondy have examined the same theory using a different approach and have reached a similar conclusion (22).

A modification of the single compartment-exponential disposal theory can be made by adding a constant term to the right side of the equation (B) to represent the concentration of plasma 17-OH-CS bound to transcortin, or it can be assumed that below a certain concentration the disposal

process follows zero rather than first-order kinetics. However, it is not worth while presenting these modifications of the theory in detail because, when they are utilized to examine the present data, the modified theories are also defective.

The failure of the single compartment-exponential disposal theory to describe adequately the disappearance of plasma 17-OH-CS has a number of consequences. Only reactions that follow first-order kinetics have half-times that are independent of concentration (23). When the term "half-time" is used in describing plasma 17-OH-CS disposal, then the dose of cortisol administered, the duration of the study and the method used in plotting the data to make the estimate of half-time must be stated if any significance is to be attached to the determination. Exceptions to the preceding generalization are studies in which different groups of subjects are compared when the same procedures have been used. When studied under the same conditions, the rate of disposal of plasma 17-OH-CS is slower in patients with hypothyroidism (24) or liver disease (10) than it is in normal subjects. However, the statement that the half-time of plasma 17-OH-CS is a certain number of minutes has no quantitative significance, since it refers to a process which does not follow first-order kinetics. Methods for quantitative measurement of adrenocortical function that utilize the assumptions of single-compartment distribution and exponential disposal of plasma 17-OH-CS (1, 5) are based on a theory which does not describe the data accurately.

Acknowledgments

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INFLUENCE OF OOPHORECTOMY ON STEROID EXCRETION IN EARLY PREGNANCY

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ABSTRACT

In a pregnant patient in whom one ovary and Fallopian tube had been extirpated previously, the remaining ovary and tube were removed (together with the corpus luteum of pregnancy) seventy-eight days after the last menstruation. Complete ablation of ovarian tissue did not influence the urinary excretion of estrone, 17β-estradiol and estriol; it resulted in a transient but significant rise in pregnane-3α,20α-diol and 17-hydroxycorticosteroid output, but 17-ketosteroid excretion remained unchanged. It is concluded that the ovarian contribution to the excretion of estrogen and pregnanediol at this stage of pregnancy is very small, or nil.

IT IS well established that early removal of the corpus luteum of pregnancy (1–3) or bilateral oophorectomy (4–8) does not interfere with the course of normal gestation, not even when carried out as early as the first or second month. Thus ovarian endocrine function is not indispensable for the maintenance of early pregnancy.

Very few reports are available in the literature on the urinary excretion of estrogen or on that of pregnanediol in oophorectomized pregnant women. Although the published reports demonstrate that the placenta is a most important source of these urinary steroids, they leave considerable doubt as to the relative importance of the ovaries in steroid production and metabolism during the first trimester of human pregnancy.

It appeared to us of interest to report a case of oophorectomy in early pregnancy, in which the urinary excretion of various steroids was estimated before and immediately after oophorectomy.

MATERIAL AND METHODS

Case report.—Mrs. A.D., aged 35, had normal deliveries in 1958 and 1959. At the age of 21 the left tube and ovary had been removed because of a pseudomucinuous kystoma ovarii. At this time the right ovary had also been resected because of small follicle cysts. The operation was followed by regular periods.

She was admitted to the hospital on May 23, 1960. Her last normal period had been on March 15. Gynecologic examination revealed a soft uterus corresponding in size to

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that of the third lunar month of pregnancy. In the right adnexal region a cystic mass (diameter 6 cm.) was found. Results of a pregnancy test (Hogben) were positive.

At laparotomy, carried out on May 30, the uterus was soft and deeply congested; its size corresponded to that seen in the tenth to eleventh week of pregnancy. The right ovary, containing a dermoid cyst and a typical corpus luteum, and the Fallopian tube were removed. No hormone therapy was instituted following operation. The postoperative course was uneventful; the patient exhibited a normal temperature throughout the whole period. She was delivered of a normal child weighing 3600 Gm. on November 28, 1960.

Histologic examination.—The specimen removed at operation was examined histologically, and showed a fully mature corpus luteum corresponding to the duration of gestation.

Collection of urine. Urine was collected daily and was stored without the addition of preservative in a deep-freeze at −17° C. until analyzed.

Methods of assay. Estrogen assays were carried out according to the method of Brown (9), with slight modifications (10, 11). Pregnanediol was determined according to the method of Klopper *et al.* (12), slightly modified (13). Corticosteroids were measured as 17-hydroxycorticosteroids (17-OH-CS) by use of the method described by Appleby *et al.* (14), as modified by Birke *et al.* (15). 17-Ketosteroids (17-KS) were estimated according to the micro-method of Vestergaard (16), with slight modifications (15).

Assessments of the reliability of the methods employed in our laboratory have been described in this Journal previously (13, 15, 17, 18). All assays reported in the present investigation were carried out in quadruplicate, *i.e.*, in duplicate by two technicians. If the mean values obtained by the two technicians differed by more than 10 per cent, the assays were repeated.

RESULTS

The results of the urinary estrogen assays are summarized in Figure 1. It can be seen that complete removal of ovarian tissue on day 78 after the last menstrual period did not influence the urinary estrogen excretion, which showed the expected continuous rise. The estrogen output around week 25 of gestation agreed with that of normally pregnant women as reported by several authors (19). Thus the data indicate that the contribution of the ovaries to the estrogen excreted by pregnant women, at least after day 78 of gestation, must be very little, if any.

The pregnanediol excretion values during the same period are compiled in Figure 2. These data indicate that laparotomy and complete removal of ovarian tissue did not result in diminished urinary excretion of pregnanediol; on the contrary, there was a significant rise in pregnanediol excretion, which returned to preoperative values on the fifth or sixth postoperative day. Thus it seems fair to conclude that the bulk, if not virtually all, of the pregnanediol excreted by this patient was derived from sources other than ovarian tissue. Since the postoperative rise in pregnanediol excretion was paralleled by a shorter but similar rise in urinary corticosteroids, as shown in Figure 3, it seems likely that this increased excretion reflected an increased adrenocortical function elicited by the stress of the operation. The

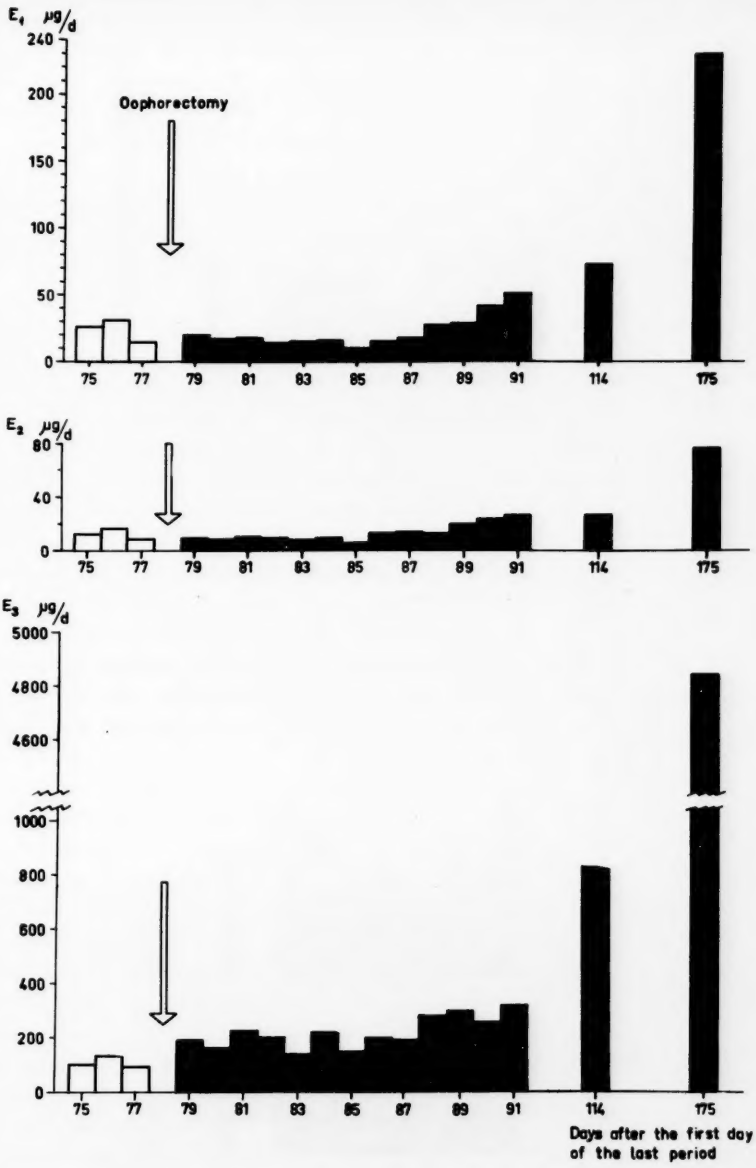

FIG. 1. Urinary excretion (µg./24 hrs.) of estrone (E_1), 17β-estradiol (E_2) and estriol (E_3) in a pregnant woman before and after complete removal of ovarian tissue. The white columns indicate preoperative excretions; the black columns indicate postoperative values.

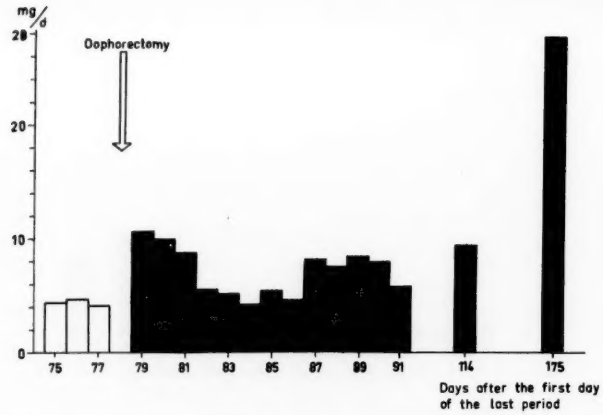

FIG. 2. Urinary excretion (mg./24 hrs.) of pregnane-3α,20α-diol in a pregnant woman before and after complete removal of ovarian tissue.

urinary 17-ketosteroid assays (Fig. 3) did not show any major changes following the complete removal of ovarian tissue.

DISCUSSION

With the exception of a paper by Rivière *et al.* (20), which was unfortunately not accessible to us but was quoted by Boute (20), all the published

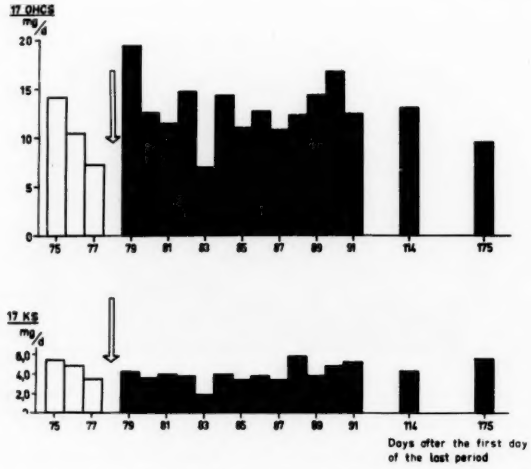

FIG. 3. Urinary excretion (mg./24 hrs.) of 17-OH-CS (upper graph) and of 17-KS (lower graph) in a pregnant woman before and after complete removal of ovarian tissue. The arrow indicates oophorectomy in each instance.

estrogen assays in bilaterally oophorectomized pregnant women were reported during the years 1928 to 1936 and were thus carried out in form of bio-assay. Such assays were reported by Amati (21), Waldstein (22), Szarka (23), Saidl (24), Allan and Dodds (25) and Guldberg (7). Oophorectomy was carried out in the individual cases between day 43 and day 112 of pregnancy, and in general it did not result in any major decrease of the estrogen content of the blood or urine. It was noted, however, in some cases, that the postoperative estrogen excretion remained at a rather low level and showed only a moderate rise during pregnancy (25). Whether or not such values were pathologically low could not be decided at that time.

Pregnanediol assays following removal of the corpus luteum of pregnancy (but not all ovarian tissue) have been reported by several groups of investigators (3, 26, 27). The gravimetric method of Venning (28) was used in the studies by Browne *et al.* (26) and in those of Jones and Weil (27). The last mentioned report is of special interest because in this case removal of the corpus luteum of pregnancy on day 58 after the last menstrual period was followed by a gradual decrease and complete disappearance of the urinary pregnanediol for approximately ten days. After this period pregnanediol could again be demonstrated in the urine although, throughout the whole pregnancy, in rather low amounts. Delivery was normal.

Tulsky and Koff (3) removed the corpus luteum graviditatis in 14 women requiring therapeutic abortion and sterilization and followed their pregnanediol excretion by the method of Guterman and Schroeder (29). Two of these women, operated upon on day 39 and day 41 of pregnancy, experienced spontaneous abortion; meanwhile the remaining 12 women (operated upon on day 35 to day 77 of pregnancy) maintained their pregnancies in a normal fashion until the uterus was emptied by the vaginal route seven to eleven days later. In the opinion of the authors, "pregnanediol urinary excretion levels obtained preoperatively and postoperatively were generally indicative of uninterrupted progesterone secretion prior to and following surgery, except in two instances in which spontaneous abortion occurred." It should be noted, however, that the day-to-day variations found by these authors exceeded considerably those reported in the present paper.

Perhaps the most informative previous report on pregnanediol excretion is the paper by Seegar and Delfs (30), because in this case all ovarian tissue had been completely removed on day 63 after the last menstrual period. They used a gravimetric method (28) but could not detect any pregnanediol in the urine excreted on the day following operation. No further assays were carried out until day 132 to day 134, when the excretion was found to be rather low (between 3.4 and 11.8 mg.). On the whole, pregnanediol excretion remained low throughout pregnancy also in this case, and only in

two instances was it higher than the values observed by these authors in normally menstruating women.

The present results on pregnanediol excretion are at variance with those of earlier reports, since we found an increase rather than a decrease following oophorectomy. Whether this depends only on differences in methodology, or in part also on other factors is difficult to decide at present.

At any rate, the data presented in this paper indicate that during the third month of pregnancy a histologically fully mature corpus luteum graviditatis may not be a quantitatively significant source of urinary estrogen and pregnanediol. This suggests that great caution is needed when histologic pictures are interpreted in functional terms. During the third month of pregnancy, chorionic gonadotropin still occurs in very high concentration in the blood and urine (31). That the major role of chorionic gonadotropin at this stage of gestation would be the stimulation of steroid production by the luteal tissue of pregnancy seems to be contradicted by the results of some of our preliminary studies (Diczfalusy and Borell, to be published), in which the administration of huge amounts of chorionic gonadotropin (100,000 I.U. daily) to pregnant women immediately before and after surgical interruption of gestation in the third month did not exert any influence on the urinary output of estrogen and pregnanediol. Thus one wonders what major endocrine role can indeed be attributed to the persisting corpus luteum of pregnancy after the first few weeks of gestation.

Acknowledgment

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THYROTROPIC ACTIVITY IN THE SERUM OF EUTHYROID, TREATED HYPERTHYROID AND POSTOPERATIVE HYPOTHYROID PATIENTS

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ABSTRACT

The bio-assay technique for thyrotropin (TSH) described by Adams and Purves and modified by McKenzie was found to have a sensitivity of 0.02 milliunit of TSH (with an injection volume of 0.5 ml.) and an average index of precision of 0.247. With this technique, TSH was detected in the sera of 16 out of 20 euthyroid subjects (0.084–0.175 mU. per ml.). The daily variation of the serum TSH level in 4 euthyroid subjects was only slight over a period of five days. Most of the thyrotropic activity (bovine TSH) added *in vitro* was recovered from the sera of euthyroid subjects and patients with Graves' disease. A rise in serum TSH concentration was noted at two and four hours after the intramuscular injection of heterologous TSH (10 USP units), with a return to the original level six hours after the injection. The maximal increase in TSH concentration was twofold. In 9 of 10 patients with Graves' disease, thyroidectomy had little effect on the serum TSH level at twenty-four and forty-eight hours after operation, but at fifteen days there was a slight decrease. By sixty days, there was an increase in serum TSH concentration in the 4 patients (of the original 10) whose sera were re-assayed. There was a delayed response (ten hours) in the thyroid activator determination with the sera of 3 out of 9 thyrotoxic patients; 2 of these 3 patients were taking antithyroid medication.

A SENSITIVE technique for the bio-assay of thyrotropin (TSH) has been described by Adams and Purves (1) and modified by McKenzie (2).

The present study confirms the sensitivity, specificity and precision of this method. It has been used to determine the rate of disappearance of exogenous TSH activity from the blood of human subjects and the effect of thyroidectomy on TSH concentration in human serum.

MATERIAL AND METHODS

Male and female SM strain mice[1] (15 Gm.), fed mice biscuit (2 mg. iodine per Kg.), were used as assay animals. Radioiodine (10 μc.) was injected intraperitoneally on the

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[1] Obtained from the National Institute of Genetics, Mishima, Shizouka Prefecture, Japan.

first day. Endogenous thyrotropin secretion was suppressed by a subcutaneous injection of stable thyroxine (10 μg.) five hours after administration of the radioiodine, repeated on the second day (5 μg.), and by the addition of desiccated thyroid (0.1 per cent) to the drinking water (Table 1). The animals were used on the fifth day for assays of the various sera and compounds to be tested for thyrotropin activity. The material was injected intravenously. Blood specimens were obtained through eye puncture by use of a glass capillary. The radioactivity in 0.1 ml. of serum (placed in 1 ml. of physiologic saline) was measured by counting the sample in a well-type scintillation counter. The probable error (PE) in counting was 1–3 per cent.

Standard thyrotropin solutions (Armour, Thytropar) were freshly made before use. The addition of 1-methyl-2 mercaptiomidazole (Merkazol, $10^{-3}M$) or potassium iodide to the TSH in sera did not influence the activity of the thyrotropin.

Blood was obtained from patients by venipuncture and refrigerated overnight before the serum was separated (2). Serum was stored at 4°C. until shortly before assay, when 0.3–0.5 ml. of serum was injected into each test mouse.

The maximal increase in blood radioactivity occurred two hours after the injection of the thyrotropin. The dose-response relationship described by McKenzie (2) was used for assay purposes, *viz*, the increase in blood radioactivity at two hours (as a percentage increase of initial activity) plotted against the dose of thyrotropin on a logarithmic scale (Fig. 1).

RESULTS

Specificity of the assay technique

In 50 experiments (6 mice each) the mean response to physiologic saline (0.2 ml.) was 110 per cent (SD ±6 per cent) of the initial blood radioactivity. In Table 2 the effects of 8 substances are compared to that of physiologic saline (in this experiment, in a single group of 10 mice). The results of administration of adrenaline (1 μg.) and cortisone (0.01 mg.) were similar to those following saline, confirming McKenzie's finding (2). Adrenocorticopin (ACTH, Organon, 1.25 I.U. or 0.4 mg.) had 1/800, and growth hormone (Armour, 5 mg.) had 1/10000 the potency of standard thyrotropin on a weight/weight basis. As suggested by McKenzie (2), these results could be explained by minimal contamination. Posterior lobe hormone (Antonin, Teizo Co., 1 unit of posterior lobe preparation of pituitary, Japanese

TABLE 1. PREPARATION OF MICE* FOR ASSAY PURPOSES

Day	Preparation
1	Intraperitoneal injection of 10 μc. of I^{131} followed by subcutaneous injection of 10 μg. of *l*-thyroxine 5 hours later.
	Addition of desiccated thyroid (0.1 per cent) to the drinking water.
2	Subcutaneous injection of 5 μg. of *l*-thyroxine.
5	Animals available for assays.

* Fed oriental diet (200 μg. of iodine/100 Gm.) throughout the experiment.

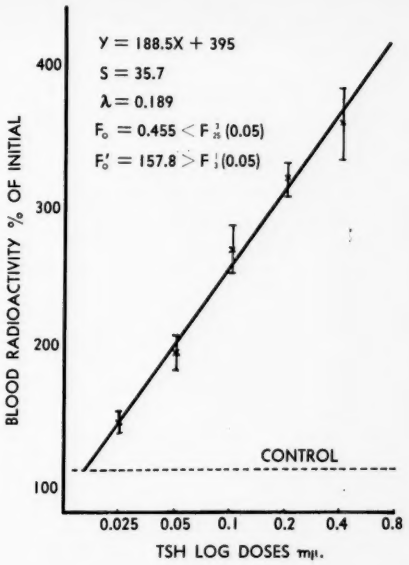

FIG. 1. Dose-response curve calculated as described by Gaddum (3).

Pharmacopoeia) also had a slight TSH-like effect. Hexestrol (synthetic estrogen, 0.1 mg.) and gonadotropic hormone (Anteron[2], Schering, 10 I.U.) had no thyrotropic effect, but chorionic gonadotropin (Primogonyl[2], Schering, 10 I.U.) had a slight TSH-like effect.

Sensitivity and precision of the assay technique (*Table 3*)

The smallest amount of thyrotropin detected by this technique was 0.02 MU. (milliunit). The range of linear responsiveness was 0.025 to 0.8 MU. (Fig. 1). The average index of precision (λ) was 0.247 for 16 assays with 2 doses of thyrotropin. The error of an assay would therefore be 26.1 per cent.

Application of the assay method

a) *TSH concentration in the sera of euthyroid subjects.* As shown in Table 4, the levels (mean) of TSH in the serum of 16 euthyroid subjects ranged from 0.084 to 0.175 mu. (This was in the winter.) In 4 euthyroid subjects, serum TSH could not be determined by this method.

b) *Recovery experiment.* Thyrotropin (0.2 MU., USP, of Thytropar, Armour) was added to a pool of serum from 10 euthyroid subjects and, in a concentration of 0.1 and 0.4 MU. per ml., to a pool of serum from 10 patients with Graves' disease treated with 0.3–0.5 Gm. of mercaptoimidazole

[2] We are grateful to Japan Schering Co. for the supply of Anteron and Primogonyl.

TABLE 2. EFFECT ON BLOOD RADIOACTIVITY OF VARIOUS SUBSTANCES ADMINISTERED INTRAVENOUSLY

No.	Test substance	Dose per animal	Serum radioactivity as % of initial level
1	Saline (physiologic)	0.2 ml.	115 ± 16.7 (SD)*
2	Adrenaline	1 μg.	122 ± 12.7
3	Cortisone	0.01 mg.	118 ± 12.2
4	ACTH (Organon)	1.25 I.U. (0.4 mg.)	453 ± 56.3
5	Growth hormone (Armour)	5 mg.	463 ± 102.5
6	Posterior pituitary hormone (Antonin, Teizo)	1 U. (J.P.)**	236 ± 27.3
7	Gonadotropic hormone from serum (Schering)	10 I.U.	123 ± 12.6
8	Chorionic gonadotropin (Schering)	10 I.U.	175 ± 30.8
9	Hexestrol	0.1 mg.	109 ± 15.1

* Standard deviation.
** 1 unit (Japanese Pharmacopoeia).

(twenty days) followed by 120 drops of Lugol's solution (ten days). The recovery was 115 per cent (mean) from the sera of euthyroid subjects, and 81 to 98 per cent from the sera of patients with Graves' disease (Table 5).

c) *Daily variation of TSH in serum.* The daily variation in 4 healthy euthyroid male subjects (tested every day at 11:30 a.m.) was usually slight but, on occasion, was as much as 100 per cent between the extremes over a five-day period (Table 6 and Fig. 2).

d) *Rate of disappearance of TSH activity from the blood in man.* TSH (10 units, USP) was given intramuscularly in 0.9 per cent (w/v) sodium chloride to 5 euthyroid subjects. Venous blood samples (20 ml.) were drawn at 0, 1, 2, 4, 6 and 20 hours. The serum was separated and cooled to 4°C. TSH activity in the sera was then bio-assayed. A rise in TSH concentration two and four hours after the intramuscular injection and a return to or toward the original level at six hours was noted in the 5 subjects studied (Table 7

TABLE 3. PRECISION OF ASSAY

No. of doses	No. of assays	*s* mean	*b* mean	(*s*/*b*)		
				Mean	S.D.	Range
2	16	56.3	247.6	0.247	0.074	0.368–0.109

Doses of thyrotropin ranged from 0.025 to 0.8 MU.
s and *b* were calculated as described by Gaddum (3).

Error of assay

The minimum variance ($V(M)$) of the logarithm of the result of the assay equals $4\lambda^2/N$. N = total number of animals in this assay.

$$V(M) = 4\lambda^2/N = 0.010168.$$

Therefore the minimum standard deviation of the logarithm of the result $= \sqrt{0.010168} = 0.1008$. Antilog $0.1008 = 1.261$. The error of assay would therefore be about 26.1 per cent.

TABLE 4. TSH CONCENTRATION IN SERUM FROM EUTHYROID SUBJECTS

Subjects		Assay design (U:S)*	Estimate of TSH (mU. per ml.)(P=0.95)*		
No.	Age & sex		Mean	Upper limit	Lower limit
1	36M	1:2	0.136	0.274	0.071
2	26M	1:2	0.130	0.215	0.064
3	24M	1:2	0.098	0.270	0.054
4	24M	1:2	0.121	0.275	0.061
5	28M	1:2	0.089	0.154	0.073
6	23M	1:2	0.109	0.301	0.059
7	22M	1:2	0.175	0.390	0.069
8	22M	1:2	0.089	0.143	0.064
9	21M	1:2	0.098	0.253	0.068
10	21M	1:2	0.148	0.294	0.072
11	20M	1:2	0.084	0.136	0.060
12	32F	1:2	0.118	0.236	0.075
13	31F	1:2	0.095	0.164	0.062
14	23F	1:2	0.103	0.172	0.084
15	22F	1:2	0.175	0.390	0.069
16	17F	1:2	0.137	0.566	0.054
17	21F	1:2	0		
18	20F	1:2	0		
19	20F	1:2	0		
20	20F	1:2	0		

* U: Unknown serum. S: Standard thyrotropin (Armour). Calculation of fiducial limits was carried out as described by Gaddum (3). "U:S 1:2" means that a 3-point assay system was used: one dose of unknown, 2 doses of standard (see ref. 3 for details). "Upper limit" and "Lower limit" are fiducial limits.

TABLE 5. STUDY OF MIXTURES OF EXOGENOUS (BOVINE) THYROTROPIN AND SERUM

Source of serum	Milliunits of thyrotropin (USP)* added per ml. of serum	Per cent recovery (P=0.95)**		
		Mean	Upper limit	Lower limit
Euthyroid subjects†	0.2	115	169	40.0
Graves' disease‡	0.1	81	123	62.8
	0.4	98	142	70.0

* Thytropar (Armour).
** Calculation of the fiducial limits was carried out as described by Gaddum (3).
† Pooled normal sera.
‡ Pooled Graves' disease sera. Patients were treated with 0.3–0.5 Gm. of mercaptoimidazol (20 days) followed by 120 drops of Lugol's solution (10 days). They were still mildly hyperthyroid clinically.

TABLE 6. DAILY VARIATION OF SERUM TSH LEVEL IN HEALTHY MALE SUBJECTS

Subjects		Concentration of TSH (milliunits/ml.) in serum					
No.	Age & Sex	Day 1	Day 2	Day 3	Day 4	Day 5	Mean ± SD*
1	21M	0.098	0.095	0.107	0.09	0.094	0.097 ± 0.0036
2	20M	0.084	0.095	0.083	0.099	0.072	0.087 ± 0.0096
3	22M	0.089	0.083	0.102	0.087	0.078	0.088 ± 0.0088
4	21M	0.148	0.107	0.083	0.069	0.095	0.1 ± 0.027

* Standard deviation.

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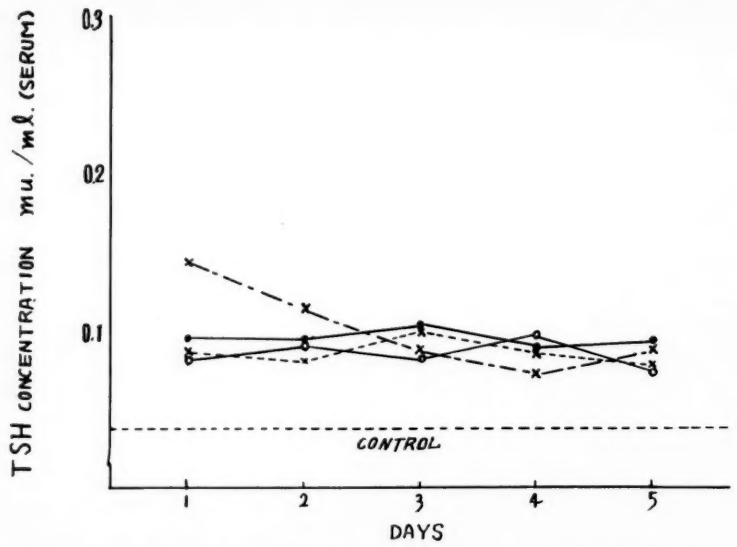

FIG. 2. Daily variation of serum TSH level in 4 healthy euthyroid male subjects. Control line represents saline injections in the control mice used for assay (2).

and Fig. 3). The maximal concentration of TSH in the serum after the intramuscular injection of 10 USP units was usually about twice the control value before injection of TSH.

e) *Effects of subtotal thyroidectomy on TSH concentration in human serum.* Ten patients with Graves' disease, treated with both mercaptoimidazole (0.3–0.5 Gm.) and Lugol's solution (120 drops) throughout the study, were thyroidectomized (subtotal). They were not given thyroid during the study following the operation. Venous blood samples (10 ml.) were drawn at 24 and 0 hours before the operation, and 24 and 48 hours after operation. In most patients there was little change in the serum TSH concentration between 24 hours before, 24 hours after, and 48 hours after operation (Fig.

TABLE 7. DISAPPEARANCE OF HETEROLOGOUS THYROTROPIN FROM THE SERUM OF 5 EUTHYROID SUBJECTS AFTER INTRAMUSCULAR INJECTION OF 10 U.S.P. UNITS OF THYTROPAR

Euthyroid subjects	Concentration of TSH (milliunits/ml.) at time-intervals					
	0	1 hr.	2 hrs.	4 hrs.	6 hrs.	20 hrs.
ME	0.130	0.135	0.164	—	0.150	0.121
KT	0.121	0.159	0.225	0.258	0.120	0.127
YG	0.109	0.117	0.194	0.200	0.121	0.101
IN	0.089	0.135	0.111	0.161	0.147	0.104
OT	0.143	0.188	0.262	0.207	0.132	0.082

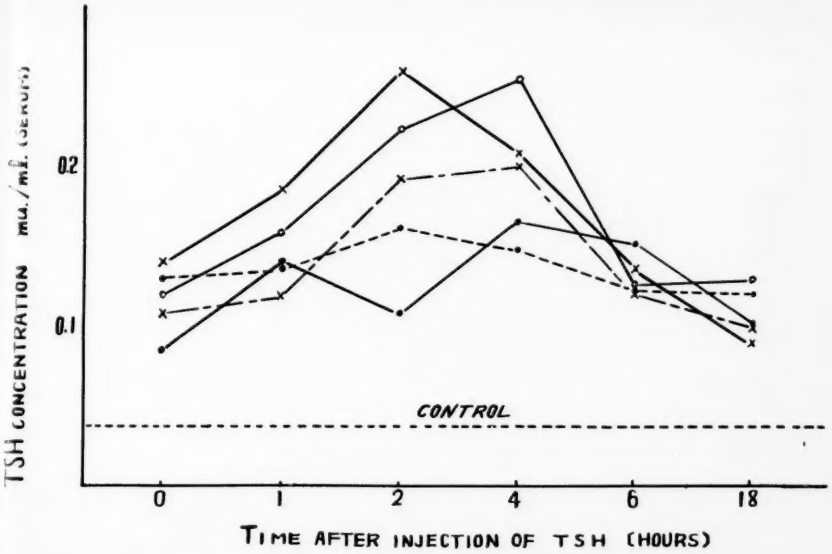

FIG. 3. Disappearance of TSH from sera of 5 euthyroid subjects after intramuscular injection of 10 units of TSH (USP). A significant rise of TSH concentration occurred at two and four hours after intramuscular injection, followed by a return to the original level at six hours. Control line represents the effect of physiologic saline injections in the control assay mice.

4), but in 3 of the 10 cases there were pronounced changes in TSH concentration at the time of the operation (the blood was drawn just prior to the injection of the local anesthetic), indicating a possible reaction to the operative preparation. There was a decrease in level of TSH at fifteen days (a mean of 0.07 mU. for the 9 patients tested compared to 0.116 mU. before operation; significant at the 5 per cent level). There was an increase of about twofold in the concentration of TSH at sixty days compared to fifteen days in the 4 patients whose sera were re-assayed (Table 8). These 4 patients were the only ones available of the original 10 and were not chosen for any particular clinical reason. Four other post-thyroidectomy patients whose sera were assayed because of the presence of mild (3 cases) or severe (1 case) hypothyroidism, had levels of TSH well above normal.

The delayed response to a thyroid activator found by Adams (4) and McKenzie (5) in the blood of patients with hyperthyroidism occurred seven to twelve hours after injection of the serum. In our study a measurement was not made during that time-interval, but at twenty-four hours the sera of 6 patients tested from among the original 10 preoperative and early postoperative patients with treated Graves' disease (Fig. 5)

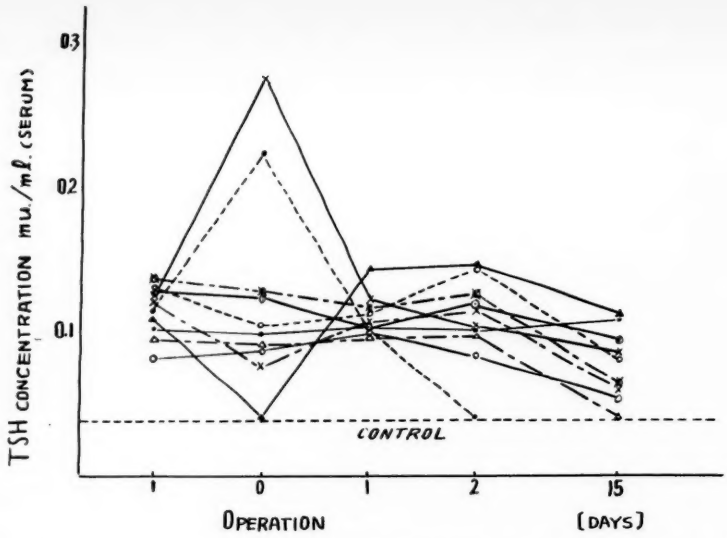

FIG. 4. Effect of thyroidectomy on TSH concentration in the sera of 10 patients with Graves' disease. In 3 cases there was a pronounced change at the time of operation (just before local anesthesia was given). The control represents the effect of physiologic saline on serum radioactivity in the control assay animals.

(As indicated in the first paragraph of Results and in Table 2, there was an increase of serum radioactivity in the assay animals in the interval following injection of physiologic saline. Each group had its own saline controls.)

TABLE 8. CONCENTRATION OF TSH IN SERUM OF PATIENTS WITH HYPOTHYROIDISM AFTER OPERATION FOR GRAVES' DISEASE

Subjects*			Days after operation	Clinical condition	BMR (%)	Estimate of TSH (mu. per ml.) (P = 0.95)		
No.	Name	Age & sex				Mean	Upper limit	Lower limit
1	AB	37F	15	euthyroid	−2	0.135	0.274	0.071
			62	mild hypothyroidism	−9	0.235	0.292	0.187
2	MT	30F	15	euthyroid	+5	0.082	0.136	0.036
			66	euthyroid	−8	0.188	0.283	0.146
3	ST	25F	15	euthyroid	+3	0.084	0.132	0.039
			72	euthyroid	−10	0.185	0.289	0.117
4	MT	32F	15	euthyroid	+3	0.122	0.275	0.049
			62	mild hypothyroidism	−9	0.250	0.589	0.064
5	SK	23F	15	euthyroid	+5	0.135	0.197	0.09
			67	severe hypothyroidism	−12	1.65	1.79	1.54
6	MM	44F	68	mild hypothyroidism	−8	1.13	1.18	1.11
7	NM	37F	1 year	mild hypothyroidism	−9	0.235	0.292	0.187
8	IR	23F	1 year	mild hypothyroidism	−8	0.420	1.11	0.22

* Patients 1–4 were among the 10 represented in Figure 4; the sera of the other 6 were not reassayed.

Patients 5–8 were not included among the 10 patients represented in Figure 4.

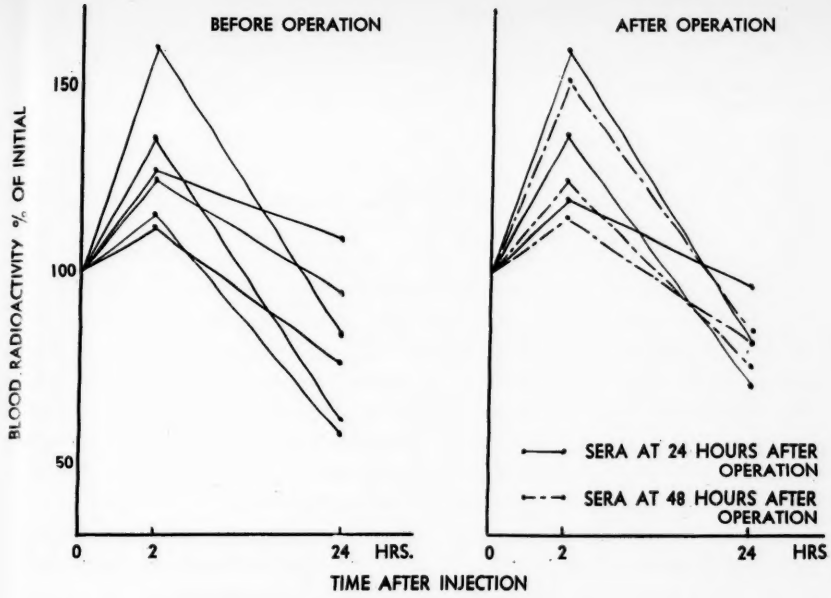

FIG. 5. Time courses of the responses to the sera of 6 preoperative and postoperative patients with treated Graves' disease.

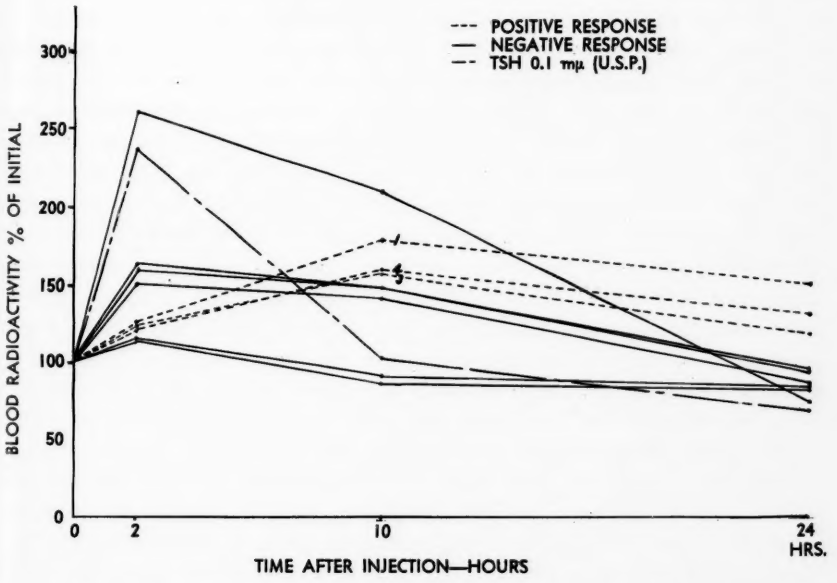

FIG. 6. Time courses of the responses to the sera of 9 patients with hyperthyroidism. Patients 1, 3 and 4 showed the long-acting thyroid stimulation effect of McKenzie (5) and Adams (4). Patients 1 and 3 were receiving antithyroid medication (Merkazol) at the time.

elicited a lower average response than did the control saline injection.

In 9 other patients with thyrotoxicosis a delayed peak response to the injection of their serum occurred at ten hours in 3 cases (Fig. 6). Two of these 3 patients had been receiving antithyroid therapy (Merkazol) and were responding satisfactorily; 2 had mild exophthalmos and 1 had normal eyes.

DISCUSSION

The average TSH concentration in normal human serum is not yet established. Although Greer and Shull (6) reasoned that there may be less than 0.06 milliunit of thyrotropin per milliliter of serum in normal human subjects, McKenzie (2) suggested that an average of about 0.2 milliunit per milliliter would be more probable. Our data showed TSH concentrations between 0.084 and 0.175 (average 0.119) mU. per ml. in the sera of 16 euthyroid subjects and no detectable amount in 4 euthyroid subjects.

Most of the thyrotropic activity was recovered from mixtures containing bovine thyrotropin (over a range of 0.1–0.4 USP milliunit per milliliter of serum) and the sera of euthyroid subjects and patients with treated Graves' disease. Interfering substances were not found.

Rapid disappearance from the plasma of large doses of heterologous thyrotropin has been reported (7). Less than 5 per cent of the administered dose of TSH remained in the rat plasma one hour after intravenous injection. The present data indicate a significant rise of TSH concentration two and four hours after intramuscular injection of thyrotropin, with a return to the original level six hours after injection. The maximal increase in concentration was only twofold with 10 USP units of TSH. It has been reported that the effect of TSH on the thyroidal accumulation of radioiodine after a single intramuscular injection into a euthyroid subject was demonstrable after a latent period of eight to ten hours, whereas an increase in the rate of release of thyroidal radioactivity had already started within the first ninety minutes (8). It is of interest that this increase in the net thyroidal accumulation of radioiodine presumably became evident about two to four hours after the serum level of TSH would have returned, in many patients, to normal. These observations suggest that the blood level of TSH determined at a given time may be quite misleading as an index of the effect TSH may be having at the tissue level at that same time.

Fifteen days after thyroidectomy (no replacement thyroid therapy) the blood level of TSH was, if anything, slightly lower than prior to operation. At thirty days, a few patients had mild symptoms of hypothyroidism. An increase in TSH concentration above the 15-day level occurred sixty days after operation in 4 patients (of the original 10), 2 of whom clinically seemed euthyroid, and 2 hypothyroid. A marked increase in TSH con-

centration was found in 4 other patients with postoperative hypothyroidism as early as sixty-seven days after surgery. Thus there may be a decrease in serum TSH concentration two weeks after thyroidectomy in patients with partially treated Graves' disease, followed by an increase in the TSH level to normal or higher. The early decrease in TSH concentration might be a response to the sudden lowering of thyroid hormone levels, leading to an initial depression in the synthesis of TSH. A continued low level of thyroid hormone might then stimulate the synthesis and release of TSH, resulting in an abnormally high serum concentration of TSH. On the other hand, recovery of function in the thyroid remnants might lead to a normal level of both thyroid hormone and TSH. It should be re-emphasized, however, that the correlation between the concentration of TSH in the blood and the amount acting at the tissue level may be poor. It is of interest, nevertheless, that the areas which produce and secrete TSH are able to respond to hypothyroidism occurring sixty days or more after thyroidectomy for Graves' disease. This suggests that, whatever the cause of hyperthyroidism, the normal mechanism for the control of TSH functions as would be predicted soon after surgical therapy.

It is unfortunate that little can be said, on the basis of the present study, regarding possible changes in the thyroid activator that might account for the delayed response in the assay technique of Adams (4) and McKenzie (5). At the time of the study it was thought that the 24-hour value would still be higher than the control value, were the long-acting thyroid stimulator present. Since then, however, Purves and Adams (9) have reported that concentration procedures may be necessary to detect this type of thyroid stimulator in patients with mild eye signs, and McKenzie (personal communication) has indicated that proper assay technique requires a determination at the seventh to twelfth hours because the 24-hour level may not be significantly different from that at the zero hour. Of 3 patients with hyperthyroidism who showed a delayed response in the determination of thyroid activator in their sera, 2 had been receiving antithyroid medication. The data, though inadequate, suggest that treatment with antithyroid drugs does not necessarily lead to disappearance of the long-acting thyroid stimulator.

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SUCCESSFUL PREGNANCY FOLLOWING HYPOPHYSECTOMY DURING THE TWELFTH WEEK OF GESTATION*

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ABSTRACT

Panhypopituitarism followed the surgical removal of a chromophobe adenoma from a woman in the twelfth week of pregnancy. The patient received hydrocortisone, desiccated thyroid and vasopressin, and the pregnancy proceeded to term in normal fashion. Minimal lactation occurred post partum. Hormonal excretion was similar to that observed in normal pregnancy. This case illustrates that, provided adequate replacement therapy is given, complete or nearly complete functional ablation of the pituitary during the latter part of the first trimester is compatible with normal human pregnancy.

THE origins of many of the hormonal changes observed during pregnancy remain obscure. In particular, evidence in the human concerning the role of the pituitary gland in producing these changes and, even more basic, in maintaining gestation, is understandably meager. Since gonadotropin deficiency accompanies panhypopituitarism and precludes ovulation, such evidence can be obtained only when pituitary deficiency develops after conception. Spontaneous pituitary necrosis is rare during pregnancy; when it occurs, fetal death has been invariable. Little and associates (1) reported that pregnancy was successful in a patient hypophysectomized during the twenty-sixth week. The present report describes endocrine studies, both before and after the delivery of a living child, in a patient whose pituitary was removed during the twelfth week of pregnancy.

METHODS

All urine collections were made in the hospital while the patient was taking 9 Gm. of salt daily. The urine specimens (collected during the period from December 10, 1959 to October 19, 1960) were frozen until assayed in October 1960. The following determina-

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tions were performed by the Bio-Science Laboratories, Los Angeles, California: *a*) urinary estrogens by the technique of Brown, as later modified (2); *b*) urinary gonadotropin, extracted by a modification of the method of Bradbury *et al.* (3) and assayed by the ovarian hyperemia response (4); and *c*) urinary aldosterone, by a physico-chemical technique (5). The aldosterone levels in 5 specimens were also measured by the double isotope derivative assay of Kliman and Peterson (6). The values by this technique were uniformly higher than, but in parallel with, those obtained by the physico-chemical assay.

The other measurements, made in the laboratory of Dr. J. W. Goldzieher, Southwest Foundation for Research and Education, San Antonio, Texas included: *a*) urinary 17-ketosteroids by a modified Zimmermann technique (7); *b*) urinary 17-hydroxycorticosteroids by the Porter-Silber method (8); and *c*) urinary pregnanediol and pregnanetriol by a method, as yet unpublished, involving enzyme hydrolysis, chromatography on silica gel and, for pregnanediol, acetylation and further chromatography on silica gel (9).

Serum protein-bound stable iodine levels were determined by the method of Barker *et al.* (10).

CASE REPORT

The patient, a 41-year-old nurse anesthetist, noticed the abrupt onset of visual blurring and frontal headache in mid-October 1959. Her last menstrual period had started on September 18, 1959, and was normal. During the next few weeks, when evidence of early pregnancy appeared, the visual disturbances and headache became more severe.

Apparently, the patient had been in normal health before October 1959. After seven years of barren marriage, she first conceived in 1955 and a normal infant was delivered by cesarian section. Lactation was profuse. In 1958, she twice miscarried at 3 months. With each pregnancy she had noticed headaches and visual blurring. These symptoms began soon after the first missed menstrual period and remained unchanged until they disappeared within three weeks after delivery.

When admitted to this hospital on December 7, 1959, she had bitemporal hemianopsia with visual acuity of 20/80 in the right eye and 20/120 in the left. Neurologic findings were otherwise normal. Abdominal and pelvic examination confirmed the presence of an intra-uterine pregnancy of about 3 months' duration. The sella turcica was enlarged and had a double floor. Erosion of the dorsum sellae and anterior clinoids was noted. The hemoglobin concentration, urinalysis findings, serum electrolyte levels, and oral glucose tolerance were all within normal limits. The results of hormonal studies are listed in Table 1.

On December 15, 1959, after institution of hydrocortisone therapy, a bulging, walnut-sized chromophobe adenoma was removed and the sella curetted. Diabetes insipidus was evident by the second postoperative day, with urine volumes between 500 and 750 ml. per hour before the start of vasopressin (Pitressin) therapy. Desiccated thyroid, 120 mg. daily, was begun on the third postoperative day, and the dosage of hydrocortisone was gradually decreased to 25 mg. daily in fractional doses.

This therapy was continued throughout her pregnancy, which was different from her previous pregnancies only in that nocturia was troublesome. This was unrelated to the intake of vasopressin. She felt well and was able to do her housework. Her libido was unchanged. Progressively less vasopressin tannate-in-oil was needed during the latter months of pregnancy; 0.2 ml. (1 unit) intramuscularly every three to six days was sufficient to keep urine volumes around 2 liters daily. In late April 1950, pedal edema de-

veloped, without arterial hypertension or proteinuria. Chlorothiazide, 500 mg. daily, was given for five days in early May 1960. A prompt diuresis, causing a 3-Kg. loss of weight in two days, was followed by a marked decrease in urine volume despite discontinuation of vasopressin for nine days.

During the last two months of pregnancy the areolae of her nipples darkened but the breasts were minimally enlarged. No lactosuria was noted by paper chromatography determination in late May 1960.

On June 10, 1960, a robust male infant weighing 7 pounds 9 ounces was delivered by an elective cesarean section without complications. After a transient increase in the dosage of hydrocortisone, the steroid and the desiccated thyroid were discontinued. During the last four days of hydrocortisone therapy (June 16 to 20, 1960), 40 units of ACTH gel were given intramuscularly. Urine flow gradually increased during the postpartum period, and a dose of 0.5 ml. (2.5 units) of vasopressin-in-oil was given on June 15, and again on June 16. On the latter day, her breasts, which had been only slightly tender and not engorged, enlarged and spontaneously secreted thin milk. Lactation diminished spontaneously and lasted only for forty-eight hours.

On returning home, she continued to require vasopressin (0.5 ml. every four to eight days), but took no other medication. Her menstrual periods did not reappear, libido disappeared, regrowth of pubic hair was slow and scanty, the skin became dry and wrinkled and appetite diminished but her weight increased and she became increasingly sensitive to cold. By late September 1960 she was easily fatigued, forgetful, mentally dull and in need of additional sleep.

Her appearance and tendon reflexes were characteristic of hypothyroidism. The vaginal mucosa was pale and atrophic and an endometrial biopsy revealed "scanty endometrium consistent with senescent changes." Visual fields and the remainder of the neurologic findings were normal. In addition to the studies detailed in Table 1, the 24-hour thyroidal uptake of radioactive iodine was 15 per cent of the administered dose on September 6, 1960.

TABLE 1. HORMONE STUDIES DURING AND AFTER PREGNANCY

	Hypophysectomy Dec. 15, '59			Delivered June 10, '60			
Date	Dec. 10, '59	Jan. 10, '60	Apr. 8, '60	May 19, '60	June 12, '60	June 22, '60	Oct. 19, '60
Week of gestation	12	17	29	35			
Therapy							
Hydrocortisone		←	—	→			
Thyroid		←	—	→			
Pitressin		←	—	→			
Urinary corticoids (mg./24 hrs.)	1.5	4.4		7.0		1.1	0.7
Urinary 17-KS (mg./24 hrs.)	5.6	8.6		17.6		3.8	3.4
Urinary aldosterone (μg./24 hrs.)	24	20	77	116	16	9	6
Urinary pregnanediol (mg./24 hrs.)	5.2	6.9		8.0		0.9	
Urinary pregnanetriol (mg./24 hrs.)	0.7	0.9		4.4		0.05	
Urinary estrogen (total) (μg./24 hrs.)	423	1,818	7,102	8,910	1,054	12	
Estrone (μg./24 hrs.)	46	25	56	56	5	5	
Estradiol (μg./24 hrs.)	19	23	46	154	9	3	
Estriol (μg./24 hrs.)	358	1,770	7,000	8,700	1,040	4	
Urinary gonadotropin (I.U./24 hrs.)	<1,000	<1,000	<1,000	>1,000 <10,000			<5 m.u.
Serum P.B.I. (μg./100 ml.)	6.1	3.6		6.7			4.0

DISCUSSION

Maintenance of pregnancy after hypophysectomy

The clinical findings and laboratory data strongly support the diagnosis of panhypopituitarism in this patient. It seems reasonable to assume that this condition followed hypophysectomy and was therefore present during the latter two thirds of her pregnancy. In order to maintain the pregnancy, hormonal replacement therapy was begun immediately after surgery and continued until delivery. Thereafter, signs and symptoms of ovarian and thyroidal deficiency promptly appeared and all tests of glandular function which were performed yielded low values. Since complete ablation was not attempted, some pituitary tissue may have remained after hypophysectomy. Even so, there is little reason to suppose that this tissue would have been functional during, but not after, pregnancy.

This patient, then, represents the first recorded case of pituitary insufficiency developing during the first trimester of a successful pregnancy. Data have been reported concerning the role of the pituitary in pregnancy in a number of animal species. Application of this information to the human is made difficult by the marked variations in the relationships between the pituitary, gonads and placenta in the various species (11). The studies of Smith upon rhesus monkeys (12) seem most applicable, since the reproductive processes of this animal and man are so similar, extending to the production of chorionic gonadotropin (13). Smith showed that pregnancy could be successful in monkeys hypophysectomized as early as the thirty-second day of a 163-day gestation. Sommers surmised from inferential data that the human subject would be relatively independent of a need for maternal gonadotropins by about the twelfth week of pregnancy (11). Our patient's experience, then, though unique, was not unexpected.

Lactation after hypophysectomy

A further similarity in the reproductive processes of the hypophysectomized human and monkey is suggested by the occurrence of lactation in this patient and in the monkeys studied by Smith. In both, secretion of milk occurred a few days after delivery; it was minimal in amount and of short duration (14). Some pituitary prolactin may have been produced by our patient, but both mammary growth and secretion can occur in the monkey deprived of this hormone (14). Ejection of milk, normally under the control of oxytocin, may also be induced by vasopressin (15), as seemed evident in this patient. Milk did not flow until the sixth postpartum day and only after the re-institution of vasopressin therapy.

Hormonal excretion during pregnancy

The changes in hormonal excretion in this patient were quite similar to

those reported for normal pregnancy (16). The increased levels of chorionic gonadotropin, estrogens and progesterone metabolites are in keeping with their recognized placental origin. The progressive rise in urinary 17-ketosteroids has been attributed to the presence of pregnanolone and other 20-ketosteroids derived from progesterone and measured by the Zimmermann reaction (16).

Urinary levels of 17-hydroxycorticosteroids, measured as Porter-Silber chromogen, increased during this patient's pregnancy while she was maintained with a constant dosage of hydrocortisone. Such an increase has not been observed in normal pregnancy (17, 18). However, a rise in the excretion of Porter-Silber chromogen similar to that noted in this patient has been observed during the course of pregnancy in some adrenalectomized women maintained with constant replacement therapy (19, 20). The mechanism for this increase is unknown. The disappearance of corticoid metabolites from the urine of such patients deprived of exogenous steroid (19, 21) seems to exclude the placenta as the source of this chromogen.

Our patient differs from the foregoing adrenal-deficient subjects, however, in that her adrenal glands were intact. Thus, if extra-pituitary adrenocorticotropic hormones appear during pregnancy, her urinary corticoid excretion would reflect their presence. Strong support for placental production of ACTH-like material has come both from assay studies (22, 23) and from the observations by Smith of the maintenance of adrenal weight and structure in his hypophysectomized monkeys during gestation (24).

A progressive increase in the excretion of aldosterone occurred in our patient, similar to that observed during normal pregnancy. This increase has been attributed, in part, to a selective increase in the excretion of that metabolite measured by the techniques used (23). However, in this and a later study (26), the secretion rate of aldosterone was also found to be increased late in pregnancy. The hypersecretion has been considered compensatory to the natriuretic effect of the elevated levels of progesterone also elaborated during gestation (27). Other studies have failed to demonstrate the placental production of aldosterone *in vitro* (28), and such production seems unlikely in view of the extremely low urinary excretion found during pregnancy in patients with Addison's disease (20, 29).

Comment

It seems, then, that pregnancy can be successfully maintained and lactation can occur in women deprived of pituitary function. The usual alterations in hormonal metabolism during pregnancy apparently depend primarily upon placental activity.

Acknowledgment

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THE DETERMINATION OF CORTISOL AND CORTICOSTERONE IN BLOOD: A REVIEW

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ABSTRACT

Methods for the determination of cortisol and corticosterone in blood are critically reviewed with special reference to work on human blood. The period covered is 1954 to the end of 1960, but some earlier work has been included. The collection and storage of blood samples, methods of extraction, purification, separation and determination are discussed in detail. Some of the more refined methods as well as the more widely used cruder techniques are reviewed in terms of available data on their sensitivity, precision, accuracy and specificity. Data are also presented on normal plasma corticosteroid levels at various times of day.

IN THE last few years considerable interest has centered on the levels of adrenocortical steroids in peripheral blood and this has been largely due to the introduction of methods which are suitable for use in routine analysis. It was hoped that such studies would reflect more accurately the concentration to which the tissues are exposed, thus providing more useful information for clinical diagnosis and investigation than that furnished by conventional estimations of hormone metabolites in the urine. It is the purpose of this review to assess the present position with regard to methodology and the results so far obtained in normal subjects. The literature has been reviewed to the end of 1960 and the scope is confined to results obtained in human subjects. Recent reviews of this topic have been published by Borth (1) and Gold (2) in 1957, and by Neher (3) in 1958.

IDENTITY OF BLOOD STEROIDS

Few investigations have been concerned with the rigorous identification of the individual steroids in peripheral human blood, and the low concentrations involved and limited volumes available have precluded the use of classic identification procedures. However, Bush and Sandberg (4) applied chromatographic methods to separate cortisol and identified this compound by several criteria including light absorption, reaction with tetrazolium salt, soda fluorescence and degradation to the expected 17-oxo-

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steroid. Evidence for the presence in human peripheral plasma of corticosterone was provided by Peterson (5), who examined its chromatographic behavior, light absorption, reaction with tetrazolium salt, alkali fluorescence and light absorption and fluorescence in sulphuric acid. Further proof of identity was offered by enzymic conversion to tetrahydrocorticosterone.

The higher steroid concentrations present in adrenal venous blood make it possible to use further identification procedures, and crystalline cortisol and corticosterone have been isolated and identified by infrared spectroscopy (6, 7). In addition there are several other reports confirming the presence of these steroids in samples of adrenal vein blood (8–12). It is not surprising that all these investigations have been made on blood samples taken during surgery from patients with various diseases, such as hypertension, hirsutism and carcinoma, but it is unlikely that any qualitative differences exist between such samples and the adrenal effluent of normal subjects. The increasing use of catheterization techniques may well provide results obtained under more physiologic conditions, but the identity of these steroids in human blood appears to be established beyond reasonable doubt.

Similar investigations have been made on blood from other species, and corticosterone or cortisol or both have been found in various proportions.

COLLECTION AND STORAGE OF SAMPLES

It has already been pointed out that the concentrations of steroid in peripheral blood are low, and in the volumes normally available for analysis only microgram or submicrogram quantities are present. It is thus particularly important from the analytical point of view that any degree of contamination or interference should be minimized and, if comparative information is sought, some standardized procedure should be adopted for the preparation of blood samples. Some variability will also occur due to the diurnal variation in blood levels, although this may be reduced by withdrawing blood at standardized times. It may be advisable to use only blood from fasting patients, in order to avoid interference by lipid material in the plasma. Attention to the cleanliness of syringes used for withdrawing blood is necessary, particularly for methods involving little purification.

Blood is usually drawn into an anticoagulant, although sometimes serum has been employed. Heparin has been widely used as an anticoagulant but some workers have preferred citrate or oxalate. There appears to be no systematic study of the effect of different anticoagulants, but it has been pointed out (13) that citrate will interfere with colorimetry in the method of Nelson and Samuels (14) and should be avoided. It would be anticipated that similar interference would occur in other methods using the Porter-

Silber reaction (15) without adequate purification. The use of serum for steroid determinations would appear to be debatable; it presupposes that no redistribution of steroid occurs between cells and plasma during the clotting process. However, Eik-Nes *et al.* (13) stated that migration of steroids into red cells takes place if blood is allowed to stand; although no experimental verification was given, this appears to have been accepted by most workers. It is, therefore, pertinent to consider the evidence available on *the distribution of cortisol between cells and plasma and the factors which influence it.*

Perkoff *et al.* (16), using the method of Nelson and Samuels (14), were unable to detect 17-hydroxycorticosteroids attached to the red cells, and Plager *et al.* (17) found only small amounts of radioactivity extractable from hemolyzed red cells by ether-chloroform (4:1) after administration of a small dose of 4-C^{14}-cortisol. Similar results were obtained by Migeon *et al.* (18) using ethanol extraction. These data conflict with those of Wu and Mason (19), who found considerable amounts of Porter-Silber chromogen in methylene-chloride extracts of frozen red cells. They used a method (20) with no chromatographic purification, and the possibility of interference by artefacts could not be excluded. However, further work by Migeon *et al.* (21) showed that, in contrast to their earlier findings (18), 17-hydroxycorticosteroids (Porter-Silber chromogenic material) could be extracted from red cells after freezing, following large doses of cortisol (100 mg. orally, or 1 mg. per Kg. of body weight intravenously). Similar results were obtained from radioactive measurements after intravenous administration of 250 μg. of C^{14}-cortisol. Migeon *et al.* (21) explain their divergent results by the preliminary washing of cells with physiologic saline in their earlier study (18), although such treatment was not mentioned in that paper. Further work with labeled steroid has been reported by Peterson *et al.* (22) who in one study injected C^{14}-cortisol, and in others added the radioactive tracer to samples of blood after withdrawal. In both cases the blood was kept at 25° and 37° C. for one and two hours; the results indicated that the erythrocytes took up cortisol. Tait (23) reported that the recovery of C^{14}-cortisol added to blood was only 80 per cent after thirty minutes (as against 95 per cent for plasma); when the blood was collected at 0° C. the recovery fell to 50 per cent.

It would therefore seem reasonable to conclude that, under the conditions of the experiments reported, cortisol is associated with the red cells, and that results to the contrary may be due to methodologic differences. It is pertinent that the proportion of steroid so associated is usually small and this would not have been detected by insensitive methods. Furthermore, Bush (24) has indicated the difficulty of extracting cortisol from lyzed cells.

However, the data so far available do not completely clarify the situation with regard to migration of steroid from plasma to cells after withdrawal of blood. It would appear that the proportion of cortisol attached to or present in the cells is fairly constant, as determined by various workers (21–23), although low temperatures may alter this radically (23). This last observation is difficult to reconcile with the findings of Wu and Mason (19) who found no change in plasma levels when oxalated blood was stored in a refrigerator for forty-eight hours, and the findings of de Moor *et al.* (25) who were able to use plasma from heparinized or oxalated samples after storing the whole blood at 4° C. overnight.

It might be expected that alterations in the distribution of cortisol would occur during clotting of blood, and in fact rather low levels have been reported in serum (26). Another group of observers consider that values obtained on plasma or serum left at room temperature reflect rapid deterioration (27). However, other workers (28, 29) have found serum levels which are within the accepted range for plasma, and a direct comparison of the serum and plasma of 5 normal subjects by the method of Nelson and Samuels (14) revealed no differences (30). It is thus clear that a more systematic investigation of the factors involved in the distribution and migration processes is highly desirable and quantitative data on this problem would be valuable. Such studies might usefully include corticosterone since, to date, information on the binding of this steroid to cells is completely lacking.

Once separated from the blood cells, the plasma can be stored in a refrigerator for a short time (31–33), or deep frozen for longer periods (32, 34, 35), with no loss of steroid. Some denaturation of protein occurs on deep freezing (36) and this may lead to difficulties when aliquots of plasma have to be withdrawn. Loss of reproducibility of results on frozen plasma has been reported when fluorometric methods were employed (25, 37).

PROTEIN BINDING

Protein binding is of considerable physiologic importance and affects steroid metabolism and excretion. Daughaday (38) has extensively reviewed the interactions between steroids and proteins.

The determination of cortisol and corticosterone is usually based on extraction from plasma with organic solvents, sometimes with, but generally without, preliminary protein precipitation. Both the bound and the unbound steroids are extractable by the solvents commonly used, so that the concentrations measured indicate the total amounts present. It is possible that protein binding reduces biologic activity and that the total plasma steroid concentrations may not, in fact, represent a measure of

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this activity at the tissue level. Possible variations in steroid binding capacity should, therefore, be taken into account when results of total plasma steroid determinations are interpreted.

EXTRACTION

General observations

At the outset it is necessary to consider whether blood, serum or plasma should be used in making steroid determinations. Although the choice will depend upon the information required, it should be borne in mind that direct extraction of the steroids from blood may give low results (24) and difficulties have been encountered in attempts to recover steroid from lyzed cells (24). It has also been claimed that extracts obtained from whole blood are not as clean as those obtained from plasma and yield less reproducible results (39). The use of serum has been discussed in a previous section; since there is some possibility that this leads to low results, it is probably better to use plasma, and most workers have done so. At present there is no evidence to indicate that precipitation of proteins offers any advantage over direct extraction, but zinc sulphate and soda (40), ethanol and zinc sulphate (41), ethanol (42–44) and acetone-methanol and magnesium chloride (34) have been employed. Since there seems to be no gross loss of steroids when precipitation is used (34, 40–42), this may be a better technique for whole blood. Basification of plasma prior to direct extraction, as recommended by Bondy *et al.* (45), causes no destruction of cortisol and is said to produce cleaner extracts.

Choice of solvents

Partition coefficients for various steroids between water and a number of organic solvents are shown in Table 1. Borth (46) has worked out useful diagrams relating the volumes of sample and extracting solvent, the number of extractions and the amount of material extracted, to partition coefficients. Suitable conditions can be found readily from such curves, but it should be remembered that complete equilibration must be ensured and that partition coefficients for plasma/solvent may differ from those for water/solvent. Thus unexpected losses may arise (47, 48). It has been shown that benzene will extract corticosterone from water more efficiently than from plasma (5).

A variety of solvents and relative volumes have been used for the extraction of plasma adrenocortical steroids—apparently on an empirical basis for the most part. The use of a 4:1 mixture of ether:chloroform (14) was based on an early observation (49) that this was better than ether for the extraction of deoxycorticosterone from urine. For the more polar steroids, particularly cortisol, at least four extractions with an equal volume are required under ideal conditions. The formation of emulsions

TABLE 1. PARTITION COEFFICIENTS (WATER/SOLVENT)

	Chloroform	Dichloromethane	Dichloroethane	Carbon tetrachloride	Benzene	Petroleum ether	Ethyl acetate	Diethyl ether
Cortisol	0.11* 0.14** 0.13†	0.13* 0.09** 0.14†	0.28†	120**	2.86* 2.7** 1.67† 3.1‡	>500**‖	0.078** 0.083†	0.77† 0.54‡
Corticosterone	0.0055**	0.0065**		0.75**	0.08** 0.1‡	190**‖	0.055**	
17-Hydroxy-11-deoxycorticosterone	<0.001§	≈0.006§		0.74§	0.11‡	≈18§¶	0.014§	
11-Deoxycorticosterone	0.06*	0.07*			0*	0.41‡#		
Cortisone	0.027**	0.033**		30**	0.9** 1.09‡	>500**‖	0.057**	0.96‡
Tetrahydrocortisone	0.12**	0.065**		75**	3.3**	>500**‖	0.048**	
Tetrahydrocortisol	0.57**	0.4**		250**	7.9**	>500**‖	0.073**	
Testosterone	<0.01**	<0.01**		0.03**	<0.01**	0.036**‖	<0.01**	
Progesterone	<0.01**	<0.01**		0.02**	<0.01**	0.01**‖	<0.01**	
Androstenedione	<0.01**	<0.01**		0.056**	0.01**	0.047**‖	<0.01**	
Prednisone	0.048**	0.042**		60**	1.3**	>500**‖	0.072**	
9α-Fluorohydrocortisone	0.23**	0.21**		265**	3.7**	>500**‖	0.042**	

* Borth, quoting L. L. Engel (46).
** Silber and Porter (67).
† Peterson (20).
‡ Carstensen (71, 96, 97).
§ 20–22° C., Braunsberg and James (156).
‖ Boiling range not stated.
¶ Boiling range, 66–71° C.
# Hexane.

with this solvent mixture is troublesome (50), and other solvents have been preferred. Good recoveries are obtained with chloroform (14), and three (50) or even two (32) extractions with 1.5 volume appear to be adequate. More recently, the use of methylene chloride has been suggested (43, 51) because of its greater stability, although the presence of phosgene in chloroform may be less deleterious (52) than was thought (13). Using one extraction with 5 volumes of methylene chloride, a theoretical yield of 98 per cent of the cortisol present in plasma can be expected, and actual recoveries from plasma have been close to this figure (20). The use of ethyl acetate (53) gives adequate recoveries of cortisol (35) and is said to yield purer extracts (54, 55), but this has been disputed (50). Isopropyl acetate has also been used (6). The use of butanol for the extraction of free steroids and the conjugates of their metabolites (40) may lead to additional complications (56), and offers no advantage when the free compounds are to be determined.

It may be concluded that methylene chloride and ethyl acetate are the solvents of choice at the present time; chloroform may be equally satisfactory.

Dialysis

Dialysis procedures have been used for the extraction of steroids from biologic fluids and tissues. They offer the theoretical advantage of providing an extract which is relatively free from lipid and pigment. Axelrod

and Zaffaroni (57, 58) studied the recovery of some adrenocortical steroids from blood, using a simultaneous extraction-dialysis method, and obtained good results (93 per cent for cortisol). Similar methods used by other workers (59–61) also provided adequate recoveries, but all these studies were made with concentrations of steroid well outside the physiologic range. Microdialysis of 1 ml. of plasma, employed by Kalant (62), resulted in "complete extraction" of 3–4 μg. of added cortisol, but Southcott *et al.* (63) obtained rather low recoveries (52–55 per cent) of 20 μg. of cortisol and corticosterone added to 50 ml. of plasma.

In practice, dialysis would appear to yield an extract which is still considerably contaminated with nonsteroidal material, so that further purification with alkali washes and solvent partition is desirable, particularly with postprandial specimens (62). Fairly long extraction times (at least six hours) are necessary, and not all workers have achieved uniformly high recoveries by this technique. It would seem, therefore, that dialysis procedures do not offer any great advantage over straightforward extraction methods.

PURIFICATION

Solvent partition

The use of a pre-wash of plasma to remove lipid with carbon tetrachloride (51), iso-octane (64, 65) or petroleum ether (66) prior to extraction, produces considerably cleaner extracts. It would appear that carbon tetrachloride leads to losses of corticosterone, whereas petroleum ether removes only the less polar C_{21} and C_{19} steroids and estrogens of low polarity (67), allowing the subsequent determination of both corticosterone and cortisol. Prednisone and 9α-fluorocortisol cannot be separated from cortisol by a single solvent partition (Table 1).

The distribution between saturated hydrocarbons and aqueous alcohols (68) has been studied by Tamm and co-workers (50, 69). One partition between equal volumes of a Shell petrol fraction similar to "Skellysolve B" and 70 per cent aqueous ethanol removed 88 per cent of plasma fats, leaving steroids of relatively low polarity in the aqueous phase. Emulsions formed when 70 per cent methanol was used (50), and more efficient purification resulted when the aqueous layer was acidified (69). Partitions between aqueous ethanol or methanol at various concentrations and ethyl acetate (42), carbon tetrachloride (42), hexane (54, 55, 70–72), pentane (53, 73, 74), toluene-hexane (73), toluene-ligroin (74) and petroleum ether (68, 75) have been used.

A benzene/water partition (76, 77) achieves considerable purification of cortisol (78) but would seem to lead to losses of corticosterone (Table 1) from the aqueous phase. The more polar estrogens, such as estriol and

the 16,17-α-ketols, when present, would remain in the cortisol fraction.

To remove some steroidal estrogens and other phenolic and acidic compounds, an alkali wash of the organic solvent extract is incorporated into some methods. One-fifth to one-tenth volume of 0.1 *N* NaOH is commonly used (47, 51) but it would seem that even treatment with *N* NaOH (11) does not lead to destruction of cortisol and corticosterone if followed by neutralization with acetic acid (35, 79). Milder conditions have been used, such as washing with sodium carbonate (4, 76) or potassium carbonate (44) or extraction from plasma after adjustment to pH 9 ± 0.5 to obviate subsequent alkali washes (45). No comparative data are available on the efficiencies of these treatments, but it would seem that no gross destruction takes place if exposure to alkali is kept to a minimum. None of these methods effectively removes the less polar estrogens, such as estrone and estradiol.

Chromatographic columns

Crude chromatographic purifications on "Florisil" (a commercial magnesium silicate preparation) (14), magnesium silicate/Celite (14), reverse phase partition (42) and silica gel (4, 6, 79) columns have been used in some methods. It is not known whether or not these offer advantages over simple solvent partitions, although it has been claimed (79) that silica gel yields purer fractions and better recovery than partitioning between petrol and 70 per cent ethanol, or reverse phase defatting.

Careful preparation of the Florisil is necessary to ensure reliable results (13, 32). Solid material interfering with the Porter-Silber reaction (15) is sometimes eluted with the steroids (14). Variable losses may occur on Florisil columns (14, 30, 32, 39) but this can be overcome to some extent by running standard columns with each batch of unknowns (14).

Recoveries of 80 per cent (80) have been obtained using columns of magnesium silicate/Celite, 1:1 (14), and some workers have adopted this technique (80–84). There was no indication in the original paper of Nelson and Samuels (14) why they used this method for whole blood but used Florisil for plasma.

Reverse phase partition columns (42) are prepared by treating kieselgur (Hyflo-Supercel) with silicone and using petroleum ether as stationary phase. Steroids are eluted with 50 per cent aqueous ethanol; fats and nonpolar material remain on the column. This method has found little application in the determination of blood steroids, but silicone-treated paper has been used successfully (35).

The preparation of silica gel columns is simple. No batch differences have been reported for these relatively crude purifications, though difficulties are encountered when finer separations are attempted (69). Satis-

factory recoveries have been reported (37) with the original method of Bush and Sandberg (4) and with a modification involving the use of benzene instead of petrol (50). It was observed with the latter method that plasma fats may contaminate the eluates and lead to poor results (50).

Comment

Most methods described for the determination of plasma cortisol and corticosterone incorporate one or more of the purification steps outlined here. The combination adopted largely depends upon the purpose of an investigation and the desired accuracy and precision. Few comparative studies have been made with respect to a given method of determination. It would seem that a pre-wash of the sample is more convenient than a solvent partition after extraction, but the relative merits of these procedures as regards purification are not known. A systematic study of the effect of alkali treatment on cortisol or corticosterone in organic solvents and the degree of purification of plasma extracts achieved may also be desirable. Among the crude chromatographic purification methods, that using silica gel offers advantages of simplicity and reproducibility, and a comparison of this with the Florisil technique may be useful. The benzene /water partition or a pre-wash of plasma with benzene may obviate the use of a chromatographic stage when only cortisol is to be determined.

SEPARATION

The separation of steroids by chromatographic methods has been dealt with in an excellent review by Neher (85). Both column and paper techniques have been applied to the determination of plasma steroids.

Column adsorption chromatography

Corticosterone and cortisol can be separated on silica gel adsorption columns (86) and this technique has been used for blood determinations (87). However, the method did not give reproducible results (34, 37, 69) and various modifications have been introduced (69, 75, 88, 89). Difficulties arising from "tailing" (69, 85) can be avoided by deactivation of the adsorbent and gradient elution (85). Although good recoveries could be obtained by adding pure steroids to such columns (75, 86) and to blood before extraction (89), the steroid fractions from plasma extracts may be contaminated with other compounds which may interfere with subsequent determination (75).

Column partition chromatography

Kieselgur partition columns were first applied to the separation of adrenocortical steroids extracted from plasma by Morris and Williams

(42). They used columns containing a stationary phase of 25 per cent aqueous ethanol with toluene as eluant to separate cortisol and cortisone. The less polar compounds were rechromatographed with the use of ethylene glycol and 20 per cent light petroleum in toluene. Although excellent recoveries of steroids were achieved by these methods, the observed high plasma concentrations of corticosterone have not been confirmed by other workers (5, 35, 37). It would seem that, at least for this compound, the degree of purification achieved is such that a highly specific method of determination or further separation from contaminants would be necessary. Many useful chromatographic systems have, however, been derived from this method (*cf.* 85) and their application to blood extracts has met with some success (37, 90).

Paper partition chromatography

Paper chromatography has been used extensively for the separation of blood steroids, and relatively crude plasma extracts have been fractionated (5, 45, 91) on Bush systems (92). The Zaffaroni (93) systems, which are capable of handling larger quantities and are thus more suitable for relatively impure extracts, offer advantages for larger blood volumes. However, the removal of the stationary phase from the paper or steroid is necessary with practically all methods of determination, and raises some problems.

The application of paper partition chromatography to the determination of the small quantities of steroids normally present in blood extracts still presents two difficulties. Firstly, the value of the "blank" becomes important and this has to be reduced by adequate washing of the paper before use. The type of treatment required is related to the method employed for the determination of the steroid. It is necessary to ensure that the "blank" is sufficiently low and uniform over the length of the paper; unfortunately, exact data are often not given in publications. Secondly, the problems of determination of the steroids *in situ* after chromatography or of satisfactory quantitative elution from paper prior to assay have not been solved and have been discussed recently (94). It would seem that for these reasons paper chromatography in most cases still leads to a lower degree of precision than does, for example, the use of partition columns. However, isotopically labeled steroid tracers, added to blood before extraction, help to obviate these difficulties (5, 91, 95).

Counter-current distribution

Counter-current distribution has been applied to extracts from adrenal vein blood (71, 96, 97) and suitable partition coefficients have been calculated for corticosterone and cortisol. No application to peripheral blood seems to have been attempted.

Comment

Adsorption chromatography for the determination of cortisol and corticosterone in blood apparently has been less successful than partition chromatography.

The relative merits of column and paper partition chromatography have been discussed recently in a useful paper by Tait and Tait (98). Under certain defined conditions the resolution obtained on paper is comparable to that on columns. Paper chromatography offers an advantage when large numbers of samples are to be analyzed. To some extent this is offset by the difficulty of attaining low or consistent blank values with some methods of determination. The supporting phases used for partition columns, on the other hand, lend themselves more readily to adequate purification. However, the careful packing, which is essential for such columns, is a slow process.

The application of counter-current separation to a large number of samples would be inconvenient and time-consuming.

DETERMINATION

The following methods are available for the determination of cortisol or corticosterone or both, after their extraction and purification:[1]

1) Measurement of light absorption at 240 mμ, typical of $\alpha\beta$ unsaturated ketones.
2) Color reactions and absorptiometric measurement. These include the condensation of carbonyl groups with hydrazines, reduction of tetrazolium salts by α-ketols or the reaction of Δ^4-3-ketosteroids with isonicotinic acid hydrazide.
3) Measurement of the fluorescence produced with alkali, sulphuric acid, or phosphoric acid.
4) Polarographic determination of derivatives.
5) Measurement of radioactivity after introduction of an isotope.

Ultraviolet absorption

The absorption peak at 240 mμ is characteristic of these and other steroids, such as aldosterone, progesterone and testosterone. Analytical difficulties may arise from reagent background absorption and from other

[1] The preparation of the sample for assay requires some care. For example, the application of heat to dry residues is undesirable, and may cause chemical change (98a), and the destructive effect of copper on steroids has been reported (98b). The ultraviolet absorption of some steroids has been employed for their localization prior to elution from paper; the destructive effect of ultraviolet irradiation may not be negligible (98c) and may invalidate the subsequent determination.

steroidal or nonsteroidal compounds with absorption in this region. Considerable purification is, therefore, necessary and extensive tests of specificity are required for a given method. The molar extinctions are lower than those produced in some color reactions (34), so that the use of larger blood volumes may be necessary. For corticosterone in human blood, 100 ml. or more would be needed under normal conditions and extensive purification is necessary (96). The method may yield good quantitative results for cortisol after adequate purification.

Color reactions

Porter and Silber (15, 47) investigated the quantitative aspects of the reaction between phenylhydrazine and steroid ketones, and their results have found extensive application to determinations in urine and blood. There is some specificity for compounds with a dihydroxyacetone group which yield derivatives with peak absorption at 410 mμ, whereas those of Δ^4-3-ketones produce peaks at about 340 mμ (47). Color development for eighteen hours at 25° C. gives more reproducible results than development for thirty minutes at 60° C. (67). When methanol is used, higher blanks may result and ethanol is preferred (47, 99). Since many compounds produce a yellow color with the sulphuric acid used in the reagent, a sulphuric acid blank with an aliquot of the sample but without the phenylhydrazine (47) is preferable to the use of a proposed color correction (14). Some discrepancies have been noted in the published values for molecular extinctions for various steroids (20, 34, 67, 100). These vary from 21,700 to 26,000 for cortisol, and an extinction (log I_0/I, where I_0 is incident, and I transmitted light intensity) of 0.05 would be obtained with a final concentration of about 0.7 μg. per ml. of reagent with a 1-cm. light path. This amount would normally be derived from 5 to 10 ml. of plasma. Microcells containing 0.5 ml. (14) or 0.2 ml. (20) with a 1-cm. light path have been used. However, a narrow cell with a longer light path, to hold 1 ml., would avoid difficulties arising from the need for very accurate measurement of so small a volume. The high precision claimed at low steroid concentrations with a method involving a final plasma equivalent of 2 ml. in 0.2 ml. of reagent (20) is indeed surprising.

Since the Porter-Silber reaction has been applied to quite crude preparations from blood (14, 47), the contribution of compounds other than cortisol must be borne in mind. Small amounts of color may arise from Δ^4-3-ketones (67), and nonsteroidal compounds (sugars, ascorbic acid, aldehydes, ketones, quinine, colchicine, potassium iodide, chloral, paraldehyde, chlorpromazine, sulphamerazine, bilirubin) may interfere (19, 56, 67, 101–103). Cortisol may account for as little as two-thirds (104) or even one-third (39) of the Porter-Silber color produced by chloroform

extracts from plasma, even when these have been submitted to crude chromatographic purification. Tetrahydrocortisone (39, 105) and other more polar steroids (39) may be present in plasma "free steroid" fractions. It would, therefore, seem unwise to equate plasma Porter-Silber chromogens with cortisol unless rigorous purification precedes determination.

A related reaction with 2,4-dinitrophenylhydrazine offers greater sensitivity but less specificity (76). Most steroid ketones produce considerable color, and absorption peaks are not sufficiently different to allow determination of individual components. Although the method may be useful after separation and careful purification, its use for crude preparations from blood (34) is not likely to yield meaningful results.

The reduction of "blue tetrazolium" (3:3′-dianisole-bis-4:4′-3:5-diphenyltetrazolium chloride) in alkaline solution (106) has been applied to the determination of cortisol and corticosterone. The reaction has been carried out with sodium hydroxide (107), choline (107), tetramethylammonium hydroxide (34, 108, 109) and other bases. Tetramethylammonium hydroxide gave the most reproducible results (34) but difficulties were encountered with some preparations (110). The quality of "blue tetrazolium" is important, and commercial samples may not be satisfactory (111). Many steroids not containing the α-ketolic grouping react more slowly (109, 112), but these are not likely to interfere with determinations of adequately purified corticosterone or cortisol. The sensitivity slightly exceeds that of the Porter-Silber reaction (34).

The blue-tetrazolium reaction has been utilized for the determination of steroids in human peripheral (113), and human (10), rabbit (70) and rat (114, 115) adrenal vein blood. Its use for crude blood extracts (34) must be condemned, since it has low specificity and is applicable only after purification on one or preferably more chromatograms. Various quantitative procedures have been adopted after paper chromatographic separations. Elution either precedes (114) or follows (116, 117) reaction of the steroid with tetrazolium salt, or the paper may be added to the reagent (113). Good results have been claimed for these methods but the sensitivity is reduced by the paper blanks. These are variable (113) and may be increased rather than reduced by solvent washing of the paper before use (114). The method is thus difficult to apply to the small amounts of steroid available from peripheral blood, and even with larger concentrations low precision has been reported (115).

The reaction of Δ^4-3-ketones with isonicotinic acid hydrazide to produce hydrazones with absorption maxima at 380 mμ (118) has been suggested as a method of determining physiologically active adrenocorticosteroids (119, 120). The sensitivity of this method is considerably lower than that using the ultraviolet absorption characteristic of these compounds. The

differences in the rates at which other ketones condense with this reagent, and in the absorption maxima, lend some specificity to the method (118), but compounds other than Δ^4-3-ketones may contribute to the reading at 380 mμ. No systematic study has been made of this method as applied to blood, and it is doubtful whether its use for relatively crude preparations (119, 120) is likely to lead to results which allow any meaningful interpretation.

Fluorometric methods

Corticosterone and cortisol undergo reactions in alkaline (92, 121) and acid (122–125) media with the formation of unknown products (125), the concentration of which can be measured fluorometrically. The availability of highly sensitive photomultipliers for this purpose makes it possible to use these methods for very dilute solutions, and the fluorescence is directly proportional to concentration when the extinction is 0.05 or less (126). This is the range of concentrations in which absorptiometric methods become inaccurate. The use of fluorometric techniques, therefore, seems attractive for work with small quantities such as those present in blood. However, these methods are more liable to interference by impurities, and careful preparation and purification of samples are essential.

The fluorescence reactions in alkaline or acid media can be applied to steroids on paper after chromatography (92, 127, 128) or in solution (121–123). A number of commercial instruments are available for fluorescence measurements in solutions (129), and suitable optical arrangements have been described for recording the soda fluorescence on paper strips (130, 131). Dependent upon the design and characteristics of the instrument, reagent blanks may or may not affect precision. Thus it would seem that the reliability of fluorometric measurements requires improvement (129, 132), for example, by better instrumentation.

Optimal conditions have been described for the development of fluorescence with sodium hydroxide on paper (92), with potassium tertiary butoxide in solution (121, 133), and with ethanolic sulphuric acid in solution (5, 132). Similar sensitivity is obtained with the alkaline and acid reagents. Fluorescence in alkali is specific for Δ^4-3-ketones (92), and fluorescence in acid is exhibited by many steroids. However, high sensitivity in the latter reaction appears to require a Δ^4-3-ketone group and a hydroxyl group at C-11 or a phenolic ring A (122, 123, 132). Some nonsteroidal compounds fluoresce in sulphuric acid (134–136) but attention to details of fluorescence development, activation and measurement may reduce such interference.

Fluorometric methods have been applied to purified fractions from human peripheral blood with variable success (5, 35, 37, 69, 75, 87, 89, 91,

137, 138), but their use for crude plasma extracts (64, 65, 139, 140) raises problems of specificity.

Polarography

An attempt to use a polarographic method for Girard T derivatives prepared from plasma steroids was made by Morris and Williams (42) but the technique has not found further application. It appears to depend upon very critical purification of each compound prior to determination; only then can quantitative results be obtained (42).

Isotopic methods

Because of the great sensitivity and precision attainable with radioactivity measurements, isotopic methods are attractive for use in plasma steroid determinations. Basically, two types of method have been employed:

1) Addition of a known amount of isotopically labeled steroid to the sample, and radioactivity measurement of the final fraction submitted to quantitative chemical analysis. This constitutes an internal standard for every sample analyzed and can be used to correct for all losses occurring during chemical manipulations.
2) Quantitative reaction of the steroid with a suitable radioactive reagent to yield a chemically stable derivative. The radioactivity of this compound after appropriate purification is a measure of the amount of steroid present.

A combination of these two methods utilizing two different and distinguishable isotopes ("double tracer technique") can offer high precision, accuracy and sensitivity, and its advantages and disadvantages have been discussed (95).

In methods of the first type, manipulations need not be quantitative and this may result in saving of time and material. Because an internal standard is used, the assessment of accuracy is more valid than that based on conventional recovery experiments. The stability of the label throughout the chemical manipulation must be ensured and a reliable chemical method of determination is still essential. Greater precision can be achieved if the quantity of tracer compound added is sufficiently small to be ignored in the final determination. However, this requires material of very high specific activity and is not always possible. The method has been applied to the determination of cortisol (45), corticosterone (5) or both (91) in peripheral blood.

In methods of the second type, the reaction used must be highly specific or a rigorous purification procedure must be adopted to isolate the

required compound. Isolation is facilitated by isotope dilution which involves addition of the inactive derivative after the radioactive compound has been formed. Conventional recovery experiments are still required for an assessment of the accuracy of these sensitive methods. Acetylation with C^{14}-acetic anhydride has been used for plasma extracts (72, 141).

The application of the double tracer technique to the determination of cortisol was reported by Avivi *et al.* (95). A C^{14}-tracer steroid was added to the plasma extract which was treated with H^3-acetic anhydride. The results were somewhat disappointing, but the availability of materials of higher specific activity together with improved instrumentation should lead to satisfactory precision, accuracy and sensitivity. Bojesen (142) used the reaction with "pipsan" (*p*-iodophenylsulphonylic acid anhydride) to determine cortisol in plasma. The extract is esterified with S^{35}-labeled reagent, and after this stage the steroid I^{131}-pipsan ester is added. Thus, the correction for losses allows for all but the initial extraction and esterification procedures.

METHODS FOR THE DETERMINATION OF ADRENOCORTICAL STEROIDS IN BLOOD: CRITERIA

Reliability

It is clearly desirable that any analytical method be assessed with the object of defining its limitations, and Borth (143) and Brown *et al.* (144) have defined general criteria for this purpose. These can be extended to cover certain problems related to blood steroid determinations and will be used to examine the reliability of available methods.

Specificity

The specificity of an analytical procedure applied to a complex mixture such as blood depends on (a) the degree of purity with which a compound to be determined is isolated, and (b) the specificity of the quantitative determination itself. Ideally (b) would be highly specific for the component to be estimated and the isolation procedure becomes less important. However, this has not been achieved for blood adrenocortical steroids, and the purity of the compounds isolated determines specificity to a large extent.

Both the purification and determination generally make use of physical and chemical properties of the compounds concerned, although some specific biologic properties can be used quantitatively. An alteration in the chemical structure of a compound leading to a change in a physical property followed by further purification can often improve separation and specificity (*e.g.*, 145, 146). Additional purification of the compound or a derivative and re-determination may serve as a further criterion of purity (*e.g.*, 5, 20). However, results on a large number of samples and under all

conditions for which a method is used would be required, and the limited amounts of blood usually available impose a severe limitation on this type of study.

Specificity can also be assessed by means of observations on animals or human subjects deficient in adrenocortical steroids (adrenalectomy, adrenal insufficiency or suppression) and on species differences (*e.g.*, rat versus man). Determinations on subjects with increased adrenal secretion (ACTH stimulation, Cushing's syndrome) offer less satisfactory evidence, since the concentrations of other compounds may increase and become measurable by the method employed.

Many color reactions are subject to interference by impurities, and certain corrections have been applied (14, 39). The validity of these corrections has not been established for the methods for which they have been used and errors may arise, particularly at low concentrations. Data presented by Bierich (78) indicate that the Allen correction (147) would lead to overestimates when used in the method of Silber and Busch (51) instead of the ethanol-sulphuric acid blank deduction suggested by the authors. A comparison of the two types of correction for 31 plasma samples (78) analyzed by a simple method including a benzene/water partition (77) gave a wider range and larger standard deviation of results about their mean when the ethanol-sulphuric acid blank deduction was used as compared with the Allen correction. Whether this is due to a difference in specificity or precision cannot be ascertained from the data.

Precision

An estimate of the precision of a method is clearly necessary for a meaningful interpretation of results. Several sources of error can be distinguished in plasma steroid determinations. Firstly, errors may arise during work-up, particularly in the more complex methods, and the precision of a method may also vary in the hands of different workers or as a result of differences in the quality of reagents and apparatus used. For example, variable losses may arise on adsorption or partition columns or paper chromatograms, or there may be variable interference from impurities. "Repeatability" has been used to indicate the precision of results obtained by a single operator using a single set of reagents and apparatus, and "reproducibility" to indicate the precision of results obtained by different operators with different sets of reagents and apparatus (148). Secondly the problem of instrumental errors may arise; the precision of a method decreases sharply as a limit of sensitivity is approached and an estimate of precision should be obtained at low concentrations.

An assessment of the precision of a given method can be made by means

of statistical analysis. Ideally, a sufficiently large number of replicate determinations would be carried out on each plasma sample and the results examined to ascertain that they do not depart seriously from a normal frequency distribution. An estimate (s) of the standard deviation (σ) of the results (x_a, x_b, x_c, $\cdots$) from their mean ($\bar{x}$) may be obtained from

$$s = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$$

where n is the number of replicate determinations.

Unfortunately it is rarely possible to carry out sufficient replicate determinations on a single plasma sample, since this requires a large volume and considerable time. If the standard deviation of individual results is likely to be constant over a reasonable range of concentrations, it is possible to use the results of duplicate determinations on a sufficiently large number of samples within this range for an estimate, s, of the standard deviation, σ, of individual results from the formula

$$s = \sqrt{\frac{\Sigma d^2}{2N}}$$

with N degrees of freedom for s, where d = difference between duplicates and N = number of duplicates (*cf.* 149). By obtaining values of s over a series of narrow ranges of concentration, any variations of the standard deviation with concentration can be detected. Bondy *et al.* (45) have stated that the difference between duplicate determinations of plasma cortisol was proportional to their mean and have worked out a proportionate standard deviation, s'. If

$$\alpha = \frac{2(x_1 - x_2)}{x_1 + x_2}$$

where x_1 and x_2 are duplicate results on the same plasma, then

$$s' = \sqrt{\frac{\Sigma \alpha^2}{2N}}.$$

However, it is only possible to use this proportionate standard deviation over the range for which it can be established from a sufficiently large number of determinations that α is independent of concentration.

Greater precision can be obtained by replicate analyses, and in general if σ is the standard deviation of individual determinations about their mean, then the standard deviation of the means of n replicates is $\sigma/\sqrt{n}$.

Accuracy

The accuracy of a method is assessed by determining the recovery of compounds added to the sample. Since the precision may vary with concentration, it is important to add amounts of steroid of the same order as those present, and even then it may not be possible to obtain an estimate of the accuracy at very low concentrations. Recoveries of 70 per cent or more are acceptable for the more complex methods, and the simpler techniques should yield 90 per cent or better. In theory it is possible to compensate exactly for losses by the addition of a radioactive tracer, but so far the counting techniques employed have not been sufficiently reliable to achieve this aim.

Sensitivity and limit of detection

If is often necessary to assess the significance of the difference between two results, and in general these results may be the means of n_1 and n_2 replicate determinations. Furthermore, the determinations may have been made using methods with differing standard deviations s_1 and s_2 (*e.g.*, 2 laboratories, or 2 operators). Provided s_1 and s_2 can be regarded as satisfactory estimates of σ_1 and σ_2 at the concentrations under consideration, the ratio

$$D \Big/ \sqrt{\frac{{\sigma_1}^2}{n_1} + \frac{{\sigma_2}^2}{n_2}} \qquad \text{(I)}$$

can be calculated, where D is the observed difference between results, and this value referred to the normal probability distribution. Thus for $P = 0.01$, the ratio must exceed 2.576; alternatively, for a given probability level, it is possible to calculate the least significant value for D.

The problem of sensitivity is of particular importance in work on plasma steroids, since it is invariably desirable to work with small volumes of blood. To overcome the lack of sensitivity of their method, Silber and Porter (47) suggested the addition of cortisol to plasma if a level below 10 μg. per 100 ml. was expected. The basic assumptions which have to be made are that the method has high precision and constant known accuracy. The results reported by Riondel *et al.* (150) using such a process were not satisfactory.

Provided sufficient data are available, it is possible to assess the limit of detection on a statistical basis. If an estimate, s, of the standard deviation can be obtained near the sensitivity limit of a method, then for n replicate determinations the least amount measurable with a maximum error of 100 per cent is

$$\frac{ts}{\sqrt{n}} \qquad \text{(II)}$$

where t is a critical value of "Student's" t corresponding to the degree of certainty required. (If s has been determined with sufficient reliability, then for a given probability level an expression can be found which depends only on n, for example, $2.576\ s/\sqrt{n}$ for $P=0.01$.) Since at this level observed values are likely to be in error by up to 100 per cent, these values have little quantitative meaning. The smallest quantity measurable with a maximal error of ± 10 per cent would be $10\ ts/\sqrt{n}$. Values of s obtained for higher concentrations cannot be used in such an assessment of sensitivity unless the standard deviation can be shown to be independent of concentration. Furthermore, if a method is to be used close to the limit of its sensitivity, adequate accuracy at low concentrations must be ensured.

At these low levels, the problem of the blank value also becomes of major importance. This has been discussed by Wilson (151) with regard to the limit of detection. Assuming that at levels close to zero, the standard deviations for estimates of the blank (s_b) and for the analytical method (s_m) are similar, then the standard deviation (s) for the corrected result will be given by $s=\sqrt{s_m^2+s_b^2}$, or $s=\sqrt{2}\ s_b$. The limit of detection may thus be defined in terms of s_b with a known probability. For example, when this limit is set at s_b, the probability of detecting this amount is 0.76; and conversely, of finding this amount when none is present is 0.24. Similarly, when a value of $3\ s_b$ is chosen, these probabilities become 0.983 and 0.017. It is thus clear that the standard deviation of the blank is of considerable importance and indeed sets a detection limit. However, these considerations are clearly invalid in cases in which for various reasons the accuracy is unacceptable at low concentrations. Also, it should be appreciated that the maximal error may still be very large at low concentrations and to minimize this error it is necessary to increase the limiting value as discussed previously. Finally, the practical difficulties involved in obtaining a true blank may be considerable, particularly with plasma samples, and since the estimate of s_b obtained from reagent blanks may be lower than the true value, s_b will not be similar to s_m. In this case the calculated limit of sensitivity must be regarded as an optimal value which will not be reached in practice, and the data obtained from an estimate of s at low levels are likely to be of more value.

These two approaches to estimating the limit of detection will yield different results, since they make different basic assumptions. Formula (II) takes no account of the variation in results which would be obtained if a plasma containing no steroid were analyzed, and assumes that such a sample would always yield a zero result (*i.e.*, its standard deviation would be zero). Wilson's procedure makes allowance for a variation at this level, and may in fact be derived from the general formula (I) as a special case in which one sample contains no steroid. Since in practice the variability of an analysis is due to technical factors unrelated to the presence or ab-

sence of steroid, the first approach would seem to be based on artificial concepts, and the higher limit of detection obtained from the second is more acceptable.

Fiducial limits

Once a satisfactory estimate of precision has been obtained, the fiducial range and error of a determination can be calculated. The fiducial range is given by $M \pm ts/\sqrt{n}$, where M is the mean of n replicates, and the maximal percentage error (with the specified degree of probability) is

$$100\ ts/M\sqrt{n}.$$

Comment

In view of the many papers now appearing which are concerned with investigation of factors which influence plasma steroid levels, it is regrettable that so few workers report sufficient data to enable an assessment to be made of the validity of the method used, or the results obtained. Indeed it would appear doubtful whether the majority of workers are prepared to allocate a small portion of their program to ensuring that their ultimate results are meaningful. Particularly in the steroid field, where analyses are seldom simple to perform and often require considerable practice, it is not justifiable to assume without investigation that a method will function adequately. It should be remembered also that the variation between laboratories, and between operators, is often considerable. Furthermore, the original data reported for some methods are often inadequate to indicate their reliability, and further results from other laboratories would be of value in making such an assessment. When methods are modified, the reasons for the modification should be given, and their effect on the performance of the method should be examined. It is clearly desirable that standard and valid statistical nomenclature and methods should be used and a sufficient number of observations made to justify the use of statistics.

Examination of the literature reveals a large number of investigations, the results of which would have to be dismissed from any critical survey simply because insufficient data are given from which their significance could be assessed. In view of the time and expense involved in conducting such studies, it would seem well justified to make some preliminary investigation of the methods to be used in relation to the problem in hand. The application of relatively simple statistical computations can often be enlightening (or discouraging) when calculation reveals the number of observations or replicate analyses which would be needed to establish a significant result.

METHODS FOR THE DETERMINATION OF ADRENOCORTICAL STEROIDS IN PERIPHERAL BLOOD

Some of the methods in use for the determination of adrenocortical steroids in blood are summarized in Tables 2 and 3. Those in Table 2 employ relatively crude extracts for assay and the specificity of the method used for determination is important. Those in Table 3 aim at determining cortisol and corticosterone individually after careful chromatographic separation.

Methods using the Porter-Silber reaction

In spite of the wide application of the Nelson-Samuels method (14), few critical studies of its reliability characteristics have been made. Considerable training (13) and care (13, 32) are required for its successful application and it would be helpful if more laboratories reported on their findings in detail.

The specificity of this method for cortisol depends largely on the validity of the corrections used to eliminate color contributed by contaminants and on the absence of other steroids containing the dihydroxyacetone group. Distortions of Porter-Silber absorption spectra have been encountered and can be reduced but not eliminated by including a benzene/water partition (77, 152) prior to Florisil chromatography. However, another group of workers stated that little extraneous color arises from plasma and reagents except for the Florisil, which gives rise to most of the background color (32). There is no evidence regarding the validity of, and errors due to, the Allen color correction (147) as used for this method (14), and some workers have preferred a sulphuric acid blank (39, 82). Some careful studies (39, 104) have shown that cortisol constitutes a variable proportion of the 17-hydroxycorticosteroids measured. The method seems to have low specificity for cortisol when used for rat plasma, since Porter-Silber chromogens were found in fractions from the plasma of abdominal aorta blood (82, 153), whereas cortisol has not been detected by other workers (37, 68).

Although good precision is indicated by a few published replicate analyses (14, 32), more data would be required for its critical assessment, particularly at physiologic levels.

The accuracy of the Nelson-Samuels procedure (14) seems to depend upon the chromatographic purification employed. Using magnesium silicate/Celite columns, recoveries of cortisol added to whole blood in amounts equivalent to 7–13 μg. per 100 ml. and taken through the whole method were 83–112 per cent in 9 experiments (14). It is not clear whether these were worked out directly from a standard curve or compared to standards

TABLE 2. METHODS FOR THE DETERMINATION OF ADRENOCORTICAL STEROIDS IN HUMAN PERIPHERAL PLASMA

Method	Sample	Extraction	Purification	Determination	Reported interference	Modifications	References for reliability data
Nelson & Samuels (14)	30 ml. blood	4×1 vol. 4:1 ether:chloroform	Hexane/70% ethanol partition and magnesium silicate/Celite columns	Porter-Silber reaction in 0.5 ml. final vol. Allen correction: 370, 410, 450 mμ			(14)
Nelson & Samuels (13, 14)	10 ml. plasma	3×1.5 vol. chloroform	Florisil columns	Porter-Silber reaction in 0.5 ml. final vol. Allen correction: 370, 410, 450 mμ	Commercial lactic acid (77)	Color blank at 410 mμ instead of Allen correction (39); addition of benzene/water partition (77)	(30, 32, 39)
Silber & Porter (47)	10 ml. plasma	1×2.5 or 5 vol. chloroform	1×2 ml. 0.1 *N* NaOH wash	Porter-Silber reaction in 1 ml. final solution by direct shaking out of washed chloroform extract. Read at 410 mμ against plasma/H_2SO_4 blank	Quinine, colchicine (56). Paraldehyde extractable with carbon tetrachloride (56). Chlorpromazine (19), bilirubin (20), acetone, bile (51)	Plasma pre-wash with carbon tetrachloride (2½ vol.) and petroleum ether (2½ vol.); substitution of methylene chloride as extracting solvent (1×2.5 vol., 1×1 vol.) (51); 1×5 vol. chloroform; use of 0.2 ml. plasma (20), addition of benzene/water partition (77) and Allen correction	(19, 20, 47)
Zenker & Bernstein (140)	0.3–3.0 ml. plasma	1×45.0 ml. chloroform	4.5 ml. 0.1 *N* NaOH	Extract chloroform with 2.4:1 sulphuric acid - 50% ethanol. Fluorescence read at 2 hrs.			
Silber *et al.* (64)	0.2–2 ml. plasma	Pre-wash plasma with petroleum ether (3 vol.); extract with methylene chloride (3 vol.)	0.2 vol. 0.1 *N* NaOH	Extraction of solvent with 30 *N* sulphuric acid and fluorescence read after 30–90 mins.			(25, 139)

TABLE 3. METHODS FOR THE DETERMINATION OF CORTICOSTERONE AND CORTISOL IN HUMAN PERIPHERAL PLASMA

Method	Sample	Extraction	Purification	Separation	Determination
Sweat (87)*†	5 ml. plasma (136)	Chloroform ? vol.	70% ethanol/petroleum ether partition	Silica gel chromatography (86)	Fluorescence in suphuric acid (122)
Morris & Williams (42)	20 ml. plasma	4 vols. ethanol	Partition in 20% ethanol/ethyl acetate and 50% ethanol/carbon tetrachloride	Celite partition chromatography	Polarography of hydrazones
Lewis (35)	5 ml. plasma	2×4 vols. ethyl acetate	Alkali wash	Paper chromatography	Fluorescence in suphuric acid
Peterson (5)	30–40 ml. plasma; add C^{14}-corticosterone	1:1 carbon tetrachloride: methylene chloride	Alkali wash	Paper chromatography	Radioactivity and fluorescecne in suphuric acid
Bondy & Upton (158); Bondy *et al.* (45)	20 ml. plasma; add C^{14}-corticosterone and C^{14}-cortisol	Add alkali; 3 vol. chloroform		Paper chromatography	Radioactivity and fluorescence in potassium t-butoxide
Braunsberg & James (37)	10 ml. plasma	2×3 vol. ethyl acetate after adjustment of plasma to pH 9±0.5 (45)	Silica gel column (4)	Celite partition chromatography	Fluorescence in ethanol sulphuric acid (5, 129)
Bojesen (142)	5–10 ml. plasma	1×1.5–3.0 vol. chloroform	70% methanol/hexane partition	Paper chromatography after esterification with "pipsan"-S^{35}	Radioactivity
Berliner (72)	5–10 ml. plasma	Chloroform ? vol.	75% methanol/hexane partition or heptane pre-wash of plasma	Paper chromatography after C^{14}-acetylation	Radioactivity
Hollander & Vinecour (141)	2 ml. plasma diluted to 12 ml. with saline	Chloroform 3×½ vol.	Alkali wash, 70% methanol/petroleum ether partition	Paper chromatography after C^{14}-acetylation	Radioactivity

* Modifications: Inclusion of alkali-wash (75); omission of petrol partition (89); modifications of eluent compositions and volumes (69, 75, 88, 89); inclusion of Florisil chromatography and omission of petrol partition (88); extraction of plasma with ethyl acetate (88), or chloroform-ethanol mixture (89); fluorescence in phosphoric acid (75).

† References for reliability data: 69, 75, 89.

taken through the chromatographic step. With the same adsorbent another group of workers reported losses of approximately 20 per cent (80), whereas "uniformly high efficiency" was claimed from still another laboratory (82). Nelson and Samuels (14), using Florisil, added cortisol to plasma in amounts equivalent to 10–40 μg. per 100 ml. and reported recoveries of 55–132 per cent for the complete method in 20 experiments. Percentages were calculated against standards taken through the chromatographic step, thus eliminating losses incurred at this stage. The most variable recoveries occurred when amounts of 10 and 20 μg. per 100 ml. were added, perhaps due to decreased precision at these levels. A further investigation (32) showed that losses arising from Florisil chromatography were 15–20 per cent, and recoveries of cortisol added to plasma in amounts equivalent to 10–60 μg. per 100 ml. (duplicate experiments) were 87–90 per cent (presumably calculated from standards taken through Florisil chromatography) in 8 experiments. Another group (30) found that recoveries of cortisol added to water and taken through the whole method increased with increasing amounts, and were 68 ± 4 per cent at a level equivalent to 30 μg. per 100 ml. At present the published results are insufficient to allow an assessment of the accuracy of this method at physiologic concentrations (*i.e.*, 5–15 μg. per 100 ml.), and errors in this range may be considerable.

The sensitivity of the method depends upon that of the Porter-Silber reaction (15, 47). With the use of 10 ml. of plasma and a final reagent volume of 0.5 ml. in a 1-cm. light path (14), the lowest measurable blood concentrations are of the order of 5 μg. per 100 ml. if 100 per cent of the cortisol present were recovered. A critical assessment of the sensitivity of the whole method is still lacking.

Although grossly increased or decreased adrenocortical activity may be reflected in results obtained by the Nelson-Samuels method (14), more refined techniques are required when precise and accurate determinations of cortisol are important.

A simple method not involving chromatographic purification and using direct extraction of cortisol from organic solvent into Porter-Silber reagent was suggested by Silber and Porter (47), and has been modified subsequently to increase its usefulness. Its specificity may be comparable to that of the Nelson-Samuels procedure (14), since although a somewhat cruder extract is used for the Porter-Silber reaction, direct extraction from the organic solvent with Porter-Silber reagent may leave considerable proportions of steroids less polar than cortisol in the organic phase (154). A comparison of results obtained with another modification (20) and an isotope dilution technique based on addition of C^{14}-labeled cortisol to plasma and determination of the specific activity of material isolated after paper

chromatography, showed excellent agreement in 6 plasma samples from normal subjects—2 samples with high, and 3 with low cortisol concentrations (20). However, the Porter-Silber reaction was used for the determination of specific activity, and the paper chromatographic system employed was unlikely to remove all tetrahydrocortisone if present. An unequivocal separation of the cortisol would provide better proof of specificity.

Good precision within the physiologic range has been claimed for a modification (20) which uses 5 ml. of plasma. However, the complete data (number of observations, range of values, method of calculating precision) were not given and a critical assessment cannot, therefore, be made. Much lower precision was reported for this type of method by another laboratory (155).

The accuracy has been reported to be as low as 30 per cent (150) but data published by other workers (20, 154) indicate an over-all recovery from plasma of 70–75 per cent. Only 60 per cent of the cortisol present was reported to be extractable from chloroform with the Porter-Silber reagent (150) but recoveries from this solvent of 94 per cent have been obtained (156), and others (20) found 90–98 per cent extractable from methylene chloride with various proportions of the reagent. To overcome gross losses, Silber and Porter (47) suggested the addition of cortisol standards to chloroform-washed plasma, followed by the usual work-up and comparison of unknowns to such "plasma standards." Later, however, Silber and Busch (51) criticized and omitted this type of procedure. The chloroform-washed plasma may not be identical with the original sample as regards such factors as partition coefficient, reagent blank, or interference with color, and the rather large plasma volumes required may not always be available. Other workers (20) employ a cortisol standard, added to water and taken through the whole method, but the use of only one standard at a concentration 5–10 times that found in plasma is not likely to yield reliable results. Nevertheless, good accuracy has been reported for this method at physiologic levels (19, 157).

The sensitivity of this method is of the same order as that of the Nelson-Samuels procedure (14), since the same method of determination is used. A smaller reagent volume (0.2 ml.) in a 1-cm. light path (20) increases sensitivity but the need for a sulphuric acid blank in this method partly cancels the improvement so that this modification is half as sensitive as the Nelson-Samuels (14) method, or 5/4 times as sensitive as the Silber-Porter (47) methods, when the different plasma volumes used are taken into account. The expedient of adding cortisol to plasma before extraction in order to overcome the low sensitivity at physiologic levels (47, 150) has been criticized in an earlier section. In a series of 16 duplicate determina-

tions on 2 ml. of plasma containing 10 μg. of 17-hydroxycorticosteroids per 100 ml. or less (mean, 5.1 μg.) the standard deviation was 1.5 μg. per 100 ml., *i.e.*, 30 per cent of the mean (150). The proportional error decreased with increasing levels of 17-hydroxycorticosteroids and the standard deviation was 6 per cent of the mean at concentrations comparable to that of the added steroid, but outside the normal physiologic range. Thus, in practice, the precision remains low at low levels, indicating that the problem of low sensitivity cannot be overcome in this way.

At present, there are not enough published results to permit a critical evaluation of the reliability of these simple methods. Much time and labor has to be expended on the cleanliness of glassware and the training of technicians (77, 155), and reports of frequent checks on recoveries and replicates are desirable. The inclusion of a benzene/water partition appears to remove much nonspecific color (78) and constitutes an alternative for, or improvement over the pre-wash of plasma (51). However, the validity of the corrections for extraneous color remains to be established.

Methods involving fluorometric determination

The acid-induced fluorescence of cortisol and corticosterone has been utilized for their determination. Simple methods analogous to that of Silber and Porter (47) have been developed, the use of fluorometry leading to much higher sensitivity. These methods have been applied to rats (64–66, 139, 140), monkeys (64), guinea pigs (64) and human subjects (25, 64).

A minimum of purification is adopted and the methods depend almost entirely upon the specificity of the fluorescence reaction. However, some residual fluorescent material (up to 6.5 μg. per 100 ml.) has been found in the plasma of adrenalectomized or hypophysectomized animals (64, 139, 140) and methods for eliminating this component have been suggested (64, 66). As much as 33 per cent of the plasma "corticosterone" measured fluorometrically may be other material (37), probably nonsteroidal. The "residual fluorescence" appears to be fairly constant, however, and if the validity of these convenient methods can be established for a given problem, they may be useful for following changes in plasma levels of cortisol or corticosterone.

Good precision has been claimed for this type of method (64, 139). Recoveries of 101–107 per cent have been reported for corticosterone added to rat plasma at levels within or above the physiologic range (64, 139) and for cortisol added to human plasma in amounts equivalent to 6–50 μg. per 100 ml. (25). The sensitivity of these methods is very high and 1–2 ml. of plasma can be used for analysis.

It would seem that these simple sensitive methods are capable of high precision and accuracy and may replace similar ones employing the Porter-Silber reaction for human blood. However, the problems of specificity and

of determining mixtures of corticosterone and cortisol should be borne in mind when results are interpreted.

Fluorometric methods of determination have also been employed after separation of cortisol and corticosterone on silica gel adsorption columns, paper partition chromatograms or Celite partition columns.

The technique involving adsorption on silica gel (86, 87, 136) has been modified and investigated in several laboratories (69, 75, 88, 89), and could be rendered more reproducible by careful purification of the chloroform used for chromatography (69). Separation of hydrocortisone and corticosterone may not be complete (88, 89) but errors arising from this were not considered to be serious. Normal levels of corticosterone in human plasma higher than those found with other methods (*cf.* 5) and the finding of hydrocortisone concentrations in rats of the same order as those in human beings (75) leave some doubt as to its specificity. The presence of unknown contaminants in the cortisol and corticosterone fractions has been demonstrated by paper chromatography (75). Replicate determinations show that with concentrations above 5 μg. per 100 ml. of plasma, satisfactory precision may be obtained (89). Standard deviations for cortisol and corticosterone were 0.4 μg. and 1 μg. per 100 ml. of plasma in 16 and 28 duplicates respectively (69); however, the mean values and ranges for this series were not given. Recoveries from the silica gel columns were 94–104 per cent for 0.25 μg. of steroids (86); >90 per cent for 0.5 μg. (75); 98–102 per cent for 0.75 μg. (75); 86–93 per cent for 1 μg. (88); and 90–100 per cent for 2 μg. (89). Recoveries of cortisol and corticosterone added to plasma were said to be 83 ± 15 per cent (69), but the concentrations used were not given; recoveries of 80–90 per cent were claimed when amounts equivalent to 80 μg./100 ml. were added to plasma (89). A method in which Florisil columns were used prior to silica gel chromatography gave recoveries of 50–70 per cent for quantities equivalent to 20 μg. per 100 ml. of plasma (88). No data are available to indicate the over-all sensitivity of the method, but precision was low at 2 μg. per 100 ml. of plasma (89).

In view of the problems of specificity and reproducibility associated with this method, its use is of limited value and other techniques for the separation of cortisol and corticosterone have yielded more promising results.

Several workers have used paper chromatographic methods for separating cortisol and corticosterone. Lewis (35) employed a preliminary reverse phase paper chromatogram to remove less polar contaminants from plasma fractions, followed by a benzene/50%-methanol chromatographic system. The steroids were determined by sulphuric acid fluorescence after elution. In other similar methods (5, 45, 158), radioactive steroids are added to the plasma to allow for losses in the isolation procedure.

The specificity of these methods depends upon the resolving power of

the paper system and the conditions used to develop the fluorescence. Peterson (5) demonstrated that the material determined as corticosterone behaved like this compound, as judged by R_f value, sulphuric acid fluoresence and absorption spectra, acetylation and reduction.

Lewis (35) found very low cortisol levels in 2 patients with Addison's disease and 1 totally adrenalectomized patient, and very low levels of corticosterone were reported by Peterson (5) in 2 patients with Addison's disease, 2 patients with hypopituitarism and in a normal subject after administration of 9α-fluorocortisol.

Twenty duplicate cortisol determinations by one method showed differences of 0–2.2 μg. per 100 ml. (35), and 75 duplicates by another method (45) gave a standard deviation of 10.8 per cent for cortisol. A further method (5) was reported to give a precision for corticosterone of ± 20 per cent at levels of 0.5 μg. per 100 ml., improving to ± 5 per cent at levels greater than 2.0 μg. per 100 ml. Recoveries from plasma were 86–96 per cent in 20 experiments with amounts of cortisol equivalent to 10–40 μg. per 100 ml., and 82–96 per cent in 10 experiments with corticosterone, the amounts added not being specified (35). The method of Bondy *et al.* (45, 158) gave recoveries of 100 ± 16 per cent for cortisol and 91 ± 13 per cent for corticosterone, both compounds having been added to plasma in rather high concentrations. The recoveries given by Peterson (5) were reported as 91–106 per cent; however, these were obtained by an unacceptable method of calculation and recalculation of the data shown gives a range of 70–115 per cent.

These paper chromatographic methods employing fluorometric determination appear to be capable of producing fairly accurate results, but their reliability at very low concentrations remains to be ascertained. It is disappointing that the use of radioactive steroids does not give more accurate results, and this may be related to errors arising from fluorometry or counting technique. Difficulties involved in eliminating the paper blank undoubtedly account for some errors, and the many attempts to cut down "background fluorescence" have not been completely successful. The use of unwashed paper leads to relatively high and variable blanks and considerable quenching may occur. Bondy *et al.* (45) found that even after extensive methanol washing, as much as 40 per cent of the total fluorescence derived from 10 ml. of plasma containing 11.7 μg. per 100 ml. of cortisol was due to the paper blank. Further data reported by Abelson (94) indicate that the mean blank value for 13.25 sq. cm. of paper was equivalent to 1.3 ± 0.06 μg. of cortisol. Peterson (5) reported no data for his blanks but stated that the values were negligible when more than 0.5 μg. of corticosterone was present in the sample analyzed. In practice this requires fairly large volumes of plasma. Lewis (35) found extremely low blank

values for alkali-washed paper; however, in contrast to other workers he eluted the paper with very small volumes (0.2 ml.) of solvent and this may partly explain the low background obtained.

It may be concluded that under normal circumstances these methods are reasonably specific for cortisol and corticosterone, but high sensitivity is achieved only when paper blanks are sufficiently low or constant. Their use for corticosterone in human plasma under normal conditions requires relatively large volumes of plasma.

The method described by Braunsberg and James (37), which involves the use of Celite partition chromatography, is lengthy and exacting. The findings of 10 μg. of corticosterone per 100 ml. of rat plasma in the absence of cortisol, and low corticosterone levels combined with cortisol concentrations of 4.8–13.3 μg. per 100 ml. of human plasma, indicate satisfactory specificity. Duplicate determinations on 7 plasma samples showed differences ranging between 0.1 and 2.2 μg. of cortisol per 100 ml. of plasma at levels between 4.8 and 15 μg. per 100 ml. Recoveries of 0.19–0.55 μg. of corticosterone from the partition columns ranged between 80 and 98 per cent; those for 0.47–1.47 μg. of cortisol were between 68 and 87 per cent. Recoveries of corticosterone added to plasma at levels equivalent to 0.5–4.7 μg. per 100 ml. were 54–83 per cent; those for cortisol at concentrations of 3.1–12.5 μg. per 100 ml. were 51–91 per cent. The fluorometric method is subject to interference by impurities and this may account for the low recoveries obtained on two occasions. For corticosterone, normal values for human subjects were less than 0.5 μg. per 100 ml.—a region in which the sensitivity of the method becomes inadequate. Concentrations of cortisol greater than 3 μg. per 100 ml. can be determined with sufficient precision. Further work is necessary before the reliability of the method can be assessed.

Methods involving determination by radioactivity

The methods of Bojesen (142), Berliner (72) and Hollander and Vinecour (141) make use of esterification of steroids with radioactive reagents and determination by radioactive counting. Insufficient data are available for an assessment of the specificity, precision and accuracy of the methods involving C^{14}-acetylation (72, 141). Contamination with other compounds is likely and much more extensive tests of specificity are required.

The principle of the method of Bojesen (142) has been outlined in an earlier section. Although it can be used for corticosterone as well as cortisol, results for cortisol only were given. The two paper chromatographic systems used for purifying the cortisol "pipsan" ester are so similar that little additional separation can be expected on the second, and an entirely different system (*e.g.*, Zaffaroni) could possibly lead to increased specific-

ity. Under the best conditions, the method is capable of good precision. Losses of cortisol through the whole method are of the order of 50 per cent but are partly corrected for by adding the labeled ester after the esterification stage. Recoveries of cortisol from human plasma were found to fall between 77 and 104 per cent. The sensitivity of the method is 4 μg. per 100 ml. when 5-ml. plasma samples are used.

These methods are potentially precise, accurate and sensitive, but they need further development and more critical tests of precision and specificity.

Normal plasma levels and diurnal variation

Tables 4 and 5 show some results for normal subjects obtained with the crude and more refined methods respectively. With one exception (159) which cannot be explained, the methods employing the Porter-Silber reaction give similar means and ranges. The techniques employing careful chromatographic separation of cortisol (Table 5) produce mean values which are not very different from those obtained by the cruder methods (Table 4); the narrower ranges reported may be partly due to the smaller numbers of samples studied. The fluorometric method (64) shown in Table 4 gives higher means, and the problem of specificity has been discussed in an earlier section. Separation of cortisol and corticosterone on silica gel appears to leave a fluorogenic impurity in the corticosterone fraction so that all corticosterone values are too high when this method of separation is combined with fluorometric determination (69, 75, 87, 89, 136, 160).

No difference between sexes could be demonstrated (25, 35, 161), although some data (91) suggest that cortisol values in women may be lower than in men. A study of larger groups of subjects of comparable ages by carefully standardized methods may or may not reveal such a difference. The day-to-day variation in normal persons has been investigated (161) and can be considerable.

Many workers have preferred to collect samples from fasting subjects. Although increased levels of plasma lipids may interfere with some of the refined techniques (37, 50), no difference was observed between fasted and unfasted subjects when a cruder method was employed (161).

It is now well established that there is a diurnal variation in the level of plasma 17-hydroxycorticosteroids in the human (36, 81, 161–164). This has been confirmed by a more specific method for cortisol (35), and corticosterone levels follow a similar pattern (165). In general, the level of circulating steroid appears to be highest around 6–8 a.m., falling to a minimum after midnight (36, 161–163), but considerable variation exists between subjects, both with regard to the peak time and the degree of fluctuation observed. No sex difference has been reported.

TABLE 4. NORMAL LEVELS OF ADRENOCORTICAL STEROIDS IN HUMAN PERIPHERAL PLASMA

Method	No. of subjects	Age (yrs.)	Sex	Time (a.m.)	Plasma adrenocortical steroids (μg./100 ml.)			Reference
					Mean	Standard deviation	Range	
Nelson & Samuels (14)	91	20–45	M	8–8.30	12	6	2–34	(161)
	29	20–45	F		15	6	2–31	
	29	64–95	?		12	6	3–24	
Nelson & Samuels (14)	40	2½–16	?	?	12	8.2	0–28.7	(173)
Nelson & Samuels (14)	16	24–45	7M, 9F	9.00	12.1	5.9	2.4–21.6	(174)
	24	Mean 22	20M, 4F		12.7	5.3	4.7–25.6	
Nelson & Samuels (14)	12	17–27	10M, 2F*	9.30	12.0	4.5	?	(175)
					12.8	4.6		
					13.8	4.7		
	12	17–27	10M, 2F*	9.30	13.5	6.2		
					12.3	6.3		
					10.8	3.6		
Nelson & Samuels, with Mg silicate-celite colums (14)	21	?	F	9.30	6.6	0.95†	?	(81)
					5.2**	0.85†		
Bayliss & Steinbeck modification (39)	10	?	F	?	9.9	2.5†	?	(176)
Silber & Porter (47)	16	?	?	?	13.3	6.2	6–25	(47)
Silber & Porter (47)	25	14–50	9M, 16F	9–11.00	12	1.1‡	4–25	(177)
Silber & Porter (47)	20	?	16M, 4F	9–10.00	16.2	4.9	6–25	(178)
Modification of method of Silber & Porter (20)	50	?	?	?	15	4.5	6–25	(20)
Modification of method of Silber & Porter (20)	30	17–40	18M, 12F	?	14	1.2†	6–25	(165)
Modification of method of Silber & Porter (20)	9	19–42	7M, 2F	?	13	4	7–22	(179)
Modification of method of Silber & Porter (20)	8	?	M	9.55	18.4	4.3	12.3–27.4	(159)
				10.30	20.5	2.0	17.3–24.2	
Modification of method of Silber & Porter (20)	90	?	?	?	13.5	3	6–25	(180)
Modification of method of Silber & Porter (51)	25	3–15	?	9–11.00	10.3	4.5	2.4–23.5	(78)
Modified Silber & Porter method (51) with benzene/water partition	31	Children	?	8–9.00	11.2§	2.9§	7.4–18.9§	(78)
					11.4‖	3.7‖	5.7–21.9‖	
Silber & Porter (47) after protein pptn.	20	23–34	M	8.30	12.7¶	0.7†¶	7.4–18.5¶	(41)
Silber *et al.* (64)	20	0–14	M	8–9.00	19.6	?	?	(25)
	14	15–24			22.1			
	23	25–39			18.4			
	55	40–59			21.4			
	24	>60			24.4			
	16	0–14	F		20.3			
	9	15–24			20.6			
	20	25–39			23.0			
	22	40–59			21.5			
	13	>60			24.5			

* Three blood samples from each subject.
** Same group, six days later.
† Presumed to be standard deviation.
‡ Standard error of the mean.
§ With H_2SO_4 blank and Allen correction.
‖ With H_2SO_4 blank only.
¶ No H_2SO_4 blank, Allen correction.

TABLE 5. NORMAL LEVELS OF CORTICOSTERONE AND CORTISOL IN HUMAN PERIPHERAL PLASMA

Author(s)	No. of subjects	Age (yrs.)	Sex	Time	Corticosterone (μg./100 ml.)		Cortisol (μg./100 ml.)	
					Mean ± S.D.	Range	Mean ± S.D.	Range
Morris & Williams (42)	7	20–45	4 M 3 F	?	7.8±2.6	4.0±10.5	8.4±1.5	6.5–10.5
Sweat (136)	6	?	?	?	4.9	4.2–5.8	11.0	7.8–12.8
Sweat (87)	21	?	M	?	4.3±2.3	?	10.8±2.6	?
Takeda (75)	17	21–30	M	?	6.1	1.4–9.6	9.2	5.3–13.6
Bojesen (142)	6	?	?	10 a.m.	—	—	13.3	6.9–20
Peterson (5)	20	?	?	?	1.1±0.3	0.5–2.0	—	—
Berliner (72)	6	?	?	3 p.m.	—	—	8.6±0.4	?
Tamm & Starlinger (69)	14	?	10 M 4 F	8:30 a.m.	1.9±1.8	0–5.5	9.5±3.1	5.6–17.8
Bondy & Upton (91)	29	?	16 M 13 F	?	1.3±2.5	<0.1–5.0	10.2±3.6	4.0–17.7
Bondy *et al.* (45)	33	?	?	?	—	—	8.1±3.6	?
Ayres *et al.* (90)	6				0.35	0–0.6	14	5–27
Lewis (35)	30	15–42	20 M 10 F	8–9 a.m.	1.2	0–6	9.2±1.5	6–12
McLaughlin *et al.* (89)	21	?	8 M 13 F		2.0	?	12.4	?
McLaughlin *et al.* (89)	9	6/52–36/52	?		10.7	?	7.4	?
Hale *et al.* (160)	44	?	M	6–10.30 a.m.	4.8±0.2	?	11.3±0.5	?
Braunsberg & James (37)	13	18–51	7 M 6 F	9.30–10.30 a.m.	—	0–0.4	7.8±2.4	4.8–13.3

Observations on animals have indicated a similar diurnal variation for monkeys (166) but this was not seen in dogs (167) or at least was less than in man (168). However, the low levels observed are near the limit of sensitivity for the method used, and the concentration changes may have been missed or may not be measurable.

The cause of such a variation in plasma levels is still obscure; it was observed in normal persons, night workers and blind subjects (164), but it has been reported that total reversal of sleep and activity for a short period (five to ten days) results in a reversal of the usual rhythm (169). Adrenal responsiveness to ACTH has been claimed to be less at night than during the day (169), thus suggesting an adrenal factor. It is of interest that patients with Cushing's syndrome with adrenal hyperplasia did not exhibit a diurnal variation (170, 171), which was also absent in another patient with an adrenal adenoma (172).

CONCLUSIONS

At present the only available methods for the separate estimation of corticosterone and cortisol which possess the required specificity are time-consuming and not easily adapted to large numbers of determinations. Simplicity can be achieved to some extent if only cortisol estimations are required, but it then becomes necessary to accept some loss of specificity. The inclusion of some adequate chromatographic stage at present appears essential if reliable estimations are to be made.

Since it is obviously desirable that determinations be made on small volumes of plasma, the question of sensitivity is of some importance. Under ideal conditions the isotopic double-labeling techniques are capable of the highest possible precision and sensitivity, but these are as yet not simple to perform, and require further development. On the other hand, the sensitivity of the simpler fluorometric methods is relatively high, and if improvements in specificity can be achieved, they may yield an acceptable assay procedure.

The development of methods for the determination of cortisol and corticosterone in plasma was prompted to a large extent by the desire to obtain a chemical measure of the hormonal activity to which tissues are subjected. More recently, however, it has become apparent that other factors such as protein binding may profoundly modify the concentration of biologically active material, and that the plasma level itself is in a continual state of variation throughout the twenty-four hours. It is thus apparent that the interpretation of plasma steroid levels must be made with caution, since hormonal activity may not be properly reflected by the concentration of circulating steroid.

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The Endocrine Society

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The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors. Endocrine Society awards are limited to citizens of the United States and Canada.

Fred Conrad Koch Award

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, Distinguished Service Professor of Physiological Chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is the highest honor of The Endocrine Society and is represented by a medal known as the Koch Medal of The Endocrine Society. The medal, as well as an honorarium of $3,500, is to be given annually to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

This Award was first presented in 1959, to Dr. W. W. Swingle and in 1960 was presented to Dr. Emil Witschi. The Koch Medal has replaced the Medal of The Endocrine Society which was established in 1954.

The Ciba Award

The Ciba Award is given in recognition of the meritorious accomplishments of an investigator in the field of basic or clinical endocrinology who has not reached his thirty-sixth birthday before June 1 of the year in which the Award is presented. This Award was established in 1942 and first presented in 1944. The Award is presently in the amount of $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $7,500, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Scholar of the Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. A grant has been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street,

Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *October 15* each year.

Proposals for appointments as a Scholar of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *October 15* each year.

Thirteenth Postgraduate Assembly in Endocrinology and Metabolism

UNDER THE CO-SPONSORSHIP OF THE ENDOCRINE SOCIETY
AND THE NATIONAL INSTITUTES OF HEALTH
BETHESDA, MARYLAND
October 2–6, 1961

A comprehensive review of clinical endocrine problems and current research activity in these areas will be presented. For further information, write to: Dr. Roy Hertz, National Institutes of Health, Building 10, Bethesda 14, Maryland. The fee will be $100.00 for physicians, with a reduction to $30.00 for Residents and Fellows. Enrollment limited to 100.

Block reservations are being held until September 15 at the following nearby motels: Bethesdan Motor Hotel, 7740 Wisconsin Ave., Bethesda, and Intown Motor Hotel, Wisconsin Ave. and Bradley Blvd., Bethesda. Registrants and faculty members must complete their own arrangements for housing by direct correspondence with these or other nearby motels. Reservations should be completed at least one month in advance.

For program, see the August issue of the Journal.

Book Register

(*The*) *Adrenal Cortex*. Edited by HENRY D. MOON, M.D., Chairman and Professor of Pathology, University of California, San Francisco Medical Center. Monograph No. 2 of the International Academy of Pathology, by 19 authors, providing lasting reference material on the structure and function of the adrenal cortex in health and disease, and related research techniques. Subjects include: The comparative anatomy, embryology, structure and function of the adrenals; biochemistry of the adrenocortical steroids; electron microscopy; mechanisms governing adrenocortical responses; diagnosis and treatment of diseases associated with the adrenal cortex; the hypothalamus and ACTH secretion; structural and biologic relationships of ACTH and MSH; infections, neoplasia, hyperfunction and hypofunction of the adrenal cortex; therapy; 315 pages; 180 figures; 18 tables; 497 references; 1961. Paul B. Hoeber, New York 16, N. Y. Price $10.50.

Adrenergic Mechanisms. Ciba Foundation Symposium, held jointly with the Biological Council's Committee for Symposia on Drug Action (Chairman, SIR CHARLES HARINGTON), the British Pharmacological Society, with the cooperation of the Physiological Society, the Biochemical Society and the Royal Society of Medicine. Edited for the Ciba Foundation by G. E. W. WOLSTENHOLME, M.B., M.R.C.P. and M. O'CONNOR, B. A., and for the British Pharmacological Society by J. R. VANE, Ph.D. The Symposium was held in the Wellcome Building, London, England, March 28–31, 1960. Among the subjects discussed by the 78 participants were: formation of adrenaline and noradrenaline, storage of catechol amines, release of sympathetic transmitter by nerve stimulation, some pharmacological properties of guanethidine, bretylium, actions of adrenaline and noradrenaline on the effector cell, a new adrenergic mechanism, synthesis of catechol amines in the depleted brain, and the awakening actions of derivatives of phenylalanine; 632 pages; 163 illustrations; 1960. Little, Brown and Company, Boston, Mass. Price $12.50.

Arteriosklerose. Ätiologie, Pathologie, Klinik und Therapie. Edited by PROF. DR. GOTTHARD SCHETTLER, Director of the Medical Clinic, Stuttgart-Bad Cannstatt and Apl. Professor, University of Tübingen, with the cooperation of PROF. DR. H. BREDT, Mainz, DR. H. HARLFINGER Tübingen, DOZ. DR. J. LINDNER, Hamburg, PROF. DR. C. ROB, London, PROF. DR. H. SAUTTER, Hamburg and PROF. DR. W. SCHULTE, Tübingen. (In German.) In this well documented study of arteriosclerosis, the definition, classification and nomenclature (*cf.* American Heart Association) and sociologic and geographic factors are considered, followed by chapters on the morphology and pathogenesis, histochemistry, the effects of growth and age on the arteries, statistical and dynamic angiochemical methods, the general etiologic factors in the development of arteriosclerosis (including the role of vessel permeability, blood constituents, infection and predisposing disease, nutrition, alcohol, tobacco and caffeine, the concentrations of plasma lipids, chylomicron size, and the presence of lipase and clearing factor), the clinical symptoms of arteriosclerosis as manifested in cardiac and renal disease and in peripheral arterial disease and diabetes, ophthalmologic changes in eye grounds, localization and combination of vascular alterations, diagnostic methods (particularly indirect procedures), ap-

pearance of arteriosclerosis after injury, and 9 chapters on therapy which cover the surgical, physical, dietary and pharmacologic aspects of treatment. Special attention is paid to the role of lipoids in arteriosclerosis. The effects of sitosterin, phenylethylacetic acid, nicotinic acid, vitamins, hormones, MER-29, heparin, adrenaline and acetylcholine in the management of arteriosclerosis are considered in detail; dietary menus are listed; 728 pages; 149 illustrations (many in color); 1960. Georg Thieme-Verlag, Stuttgart. In the U.S.A. and Canada: Intercontinental Medical Book Corporation, New York 16, N. Y. DM 98; $24.50.

Biochemistry of Steroids. By ERICH HEFTMANN and ERICH MOSETTIG, the Steroid Section, Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland. This college textbook contains chapters on cholesterol, sterols, the vitamin D group, steroid sapogenins and alkaloids, cardiac glycosides, bile acids, progesterone, corticosteroids, androgens, and estrogens. The final chapter provides a condensed list of bibliographic references; 231 pages; 110 figures; 1961. Reinhold Publishing Corp., New York; Chapman and Hall Ltd., London. Price $6.25.

Cell function. An Introduction to the Physiology of the Cell and Its Role in the Intact Organism. By L. L. LANGLEY, PH.D., LL.B., Professor of Physiology, University of Alabama, Birmingham, Ala., and illustrated by FRANCES LANGLEY. In this textbook Part I covers the functional anatomy of the cell, Part II describes intracellular activities (metabolism, movement, electrical activity, reproduction, growth, aging, and the effects of radiation), Part III deals with the integration of the cell in the total organism (tissues, and the nervous, circulatory, respiratory, urinary and endocrine systems), and Part IV provides basic background material for reference; 377 pages; 117 figures; 18 tables; 1961. Reinhold Publishing Corp., New York; Chapman and Hall Ltd., London. Price $7.50.

Ciba Foundation Study Group. No. 7. Virus Meningo-Encephalitis. In honor of PROF. K. TODOROVIĆ, Rector of the Medical Faculty, University of Belgrade. Edited by G. E. W. WOLSTENHOLME, O.B.E. and M. P. CAMERON, M.A. for the Ciba Foundation. Twenty-three participants engaged in a study group (September 27, 1960) concerning a general survey of meningo-encephalitis from the clinical standpoint, viruses as a cause of meningo-encephalitis, ECHO viruses and their clinical aspects, histopathology and clinical aspects of Coxsackie and Coxsackie-like viruses, polio as a cause of meningitis and meningo-encephalitis, the tick-borne encephalitis complex, arbo-virus encephalitis, significance of inclusion bodies in virus meningo-encephalitis, post-infectious virus encephalitis, and neurologic complications of Behcet's disease; 120 pages; 11 illustrations; 1961. Little, Brown and Company, Boston, Mass.

Control of Ovulation. Proceedings of the conference held at Endicott House, Dedham, Mass., 1960. Edited by CLAUDE A. VILLEE, Harvard Medical School, Boston, Mass. Subjects covered by the conference were: the role of the pituitary gonadotropins in induction of ovulation in the hypophysectomized rat; follicular development, ovular maturation and ovulation in ovarian tissue transplanted to the eye; the role of steroids in the control of mammalian ovulation; the pituitary stalk and ovulation; interactions between the CNS and hormones influencing ovulation; the preoptic region of the brain and its relation to ovulation; mechanisms controlling ovulation of avian and mammalian follicles; ovulation in the domestic fowl; hormonal augmentation of fertility in sheep and

cattle; the induction of ovulation in the human by human pituitary gonadotropin; factors influencing ovulation and atresia of ovarian follicles; and the inhibition of ovulation in the human. The results of current attempts to control ovulation by the administration of analogues of the steroid hormones are discussed in detail; 251 pages; 126 figures; 49 tables; 460 references; 1961. Pergamon Press, Inc., New York and London. Price $10.00.

(*The*) *Encyclopedia of the Biological Sciences.* Edited by PETER GRAY, Head, Department of Biological Sciences, University of Pittsburgh. This is a comprehensive reference source (not merely a dictionary) which contains over 800 articles contributed by experts from 30 countries. It covers the whole spectrum of the biological sciences. All articles define, describe and explain their subjects. There is good balance between modern and classic presentations. The aim is to provide maximal information for the greatest possible number of people. Developmental, ecological, functional, genetic, structural and taxonomic aspects are considered. Numerous topics in the fields of biophysics and biochemistry are covered, as well as biographies. A full index is included; 1119 pages; illustrated; 1961. Reinhold Publishing Corp., New York; Chapman and Hall, Ltd., London. Price $20.00.

Essential Hypertension. The Proceedings of an International Symposium sponsored by CIBA, and held in Berne, Switzerland, June 7–10, 1960, (Chairman: F. C. REUBI). Edited by K. D. BOCK, Basle, and P. T. COTTIER, Berne. Forty specialists from twelve countries presented papers or discussed two specific questions of interest in the study of essential hypertension. These are: the relationship between essential hypertension and salt and water metabolism, and the long-term effects of antihypertensive therapy. The aspects of these problems include the mosaic theory of hypertension, inheritance of high blood pressure, nature of essential hypertension, the possible role of salt intake on the development of hypertension, renal hemodynamics, water and electrolyte excretion in essential hypertension, adrenocortical function and renal pressor mechanisms in experimental hypertension, relationships between salt metabolism and the angiotensin system, adrenocortical function in essential hypertension, significance of hyperaldosteronuria, therapeutic aspects of salt restriction, mechanism of hypotensive action of saluretics, the natural history of benign hypertension, the natural course of malignant hypertension, pharmacology of new hypotensive drugs, bretylium and guanethidine (clinical results), combined drug therapy in hypertension, effects of the administration of saluretic drugs on the treatment of arterial hypertension, survival rates in severe hypertension intensively treated with hydralazine and ganglionic blockade, late effects of hypotensive drug therapy on renal function in essential hypertension, late results of surgical therapy (sympathectomy and adrenalectomy), hypertension and its associated vascular diseases, and the prevention and treatment of "atheromatous complications in hypertension; 392 pages; 81 figures; 62 tables; 1960. Springer-Verlag, Berlin.

Famous Faces in Diabetes. Compiled by CECIL STRIKER, M.D., First President, American Diabetes Association; Assistant Clinical Professor of Medicine, University of Cincinnati; Attending Physician, Cincinnati General Hospital and Jewish Hospital. Foreword by Elliott P. Joslin, M.D. This book contains pictures and biographical data of the men who have made significant contributions to the subject of diabetes. The entire history of the syndrome is covered; 256 pages, 201 illustrations; 1961. G. K. Hall and Company, Boston 10, Mass. Price $25.00.

Foetal and Neonatal Physiology. British Medical Bulletin, Volume 17, Number 2. Scientific Editor, PROF. K. W. CROSS, Professor of Physiology, London Hospital Medical College. Chairman of the Symposium Committee, PROF. J. H. BURN. As stated in the Introduction by DR. R. A. MCCANCE, Professor of Experimental Medicine, University of Cambridge: "There are many working actively today on the subjects of foetal and neonatal physiology. The problems are being studied by men and women in India, Russia, the United States of America, Mexico, Jamaica, Uganda and South Africa. Great advances will surely follow, and judging by the contents of this number Great Britain is ready to play a leading part." The subjects covered are: Histology of the Placenta, Foetal Endocrinology, Protein Transfer across Foetal Membranes, Immunological Tolerance and Paralysis, Transport Mechanisms in the Foetus, Foetal Kidney, Enzymic Activity during Early Development, Carbohydrate Metabolism in Placenta and Foetus, Inferior Aortico-Pulmonary Glomus, Prenatal Mineral Metabolism, Glycogen Reserves and Their Changes at Birth, Ability of Young Mammals to Withstand Total Oxygen Lack, Changes in the Circulation at Birth, Blood Pressure and Respiration in the New-Born Baby, The Newborn Animal and Environmental Temperature, and Neonatal Paediatrics and Animal Physiology; 98 pages; illustrated; 1961. The Medical Department, The British Council, 65 Davies Street, London, W. 1, England. Price $3.25.

Highlights of Research Progress in Allergy and Infectious Diseases, 1960. Prepared by the NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES as background material for submission at congressional hearings on appropriations for fiscal year 1962. Public Health Service Publication No. 829, Summarizing work in the fields of allergy-immunology, and virus, parasitic, bacterial, fungus and rickettsial diseases; 71 pages; 1961. U. S. Department of Health, Education, and Welfare, Bethesda, Maryland. Price 30 cents.

Metabolic Effects of Adrenal Hormones. Ciba Foundation Study Group, No. 6. (Chairman, F. G. YOUNG, Dept. of Biochemistry, University of Cambridge). In honor of Professor G. W. THORN, Department of Medicine, Peter Bent Brigham Hospital, Boston, Massachusetts. Edited by G. E. W. WOLSTENHOLME, and MAEVE O'CONNOR. Twenty-seven participants in a study group, held in London, England, July 15, 1960, discussed the following topics: Actions of cortisol and related compounds on carbohydrate and protein metabolism; the rôle of adrenal steroids in the regulation of hepatic metabolism; the adrenal gland and *in vitro* protein synthesis; action of cortisol on trapping of amino acids by the liver; and the effect of adrenal hormones on adipose tissue; 109 pages; 16 figures; 16 tables; 1960. Little, Brown & Company, Boston, Mass.

L'Année endocrinologique, Thirteenth year. By M. ALBEAUX-FERNET, L. BELLOT, J. CHABOT, J. DERIBREUX, M. GELINET and J.-D. ROMANI. (In French). This yearly volume contains articles dealing with the CNS control of the adrenal cortex; spirolactones as antagonists of aldosterone; possible autonomy of hyperthyroidism; uptake and dosage of I^{131}, and treatment of cardiac complications in hyperthyroidism; recent work on the parathyroid hormone, parathyroid bone disease, and hypoparathyroidism; gonadal dysgenesis; detection of diabetes by various tolerance tests; role of the liver in hormonal metabolism; anabolic steroids with weak androgenic effects; efficacy of the buccal route for anticontraceptives; and medical and surgical problems in hermaphrodism; paper; 221 pages; 40 illustrations; 1961. Masson et Cie, Paris 6, France.

National Institutes of Health. Scientific Directory, 1961, and Annual Bibliography, 1960.

The directory reflects organizational structure and position incumbents of the National Institutes of Health. The bibliography enumerates papers published by the staff and the journals they appeared in. Both author and subject indexes are provided; 144 pages; 1961. U. S. Department of Health, Education, and Welfare, Bethesda, Maryland. Price 45 cents.

Progress in Medical Genetics, Volume I. Edited by ARTHUR G. STEINBERG, PH.D., Professor of Biology and Associate Professor of Human Genetics, Western Reserve University, Cleveland, Ohio. Developments in the field are discussed by the following authorities: Mutation in man (J. F. Crow); natural selection in man (J. B. S. Haldane); genetics and congenital malformations (F. C. Fraser); blood groups and disease (C. A. Clarke); ABO incompatibility (H. Levene and R. Rosenfield); the hemoglobinopathies (D. Rucknagel and J. Neel); morbidity of children from consanguineous marriages (N. E. Morton), and chromosomes and human disease (M. Ferguson-Smith); 341 pages; 29 figures; 64 tables; 1086 references; 1961. Grune and Stratton, New York 16, N. Y. Price $9.75.

Quinones in Electron Transport. A Ciba Foundation Symposium edited by G. E. W. WOLSTENHOLME, O.B.E. and C. M. O'CONNOR, B.Sc. The symposium held May 11–13, 1960 in London, was attended by 29 pioneers and experts who discussed: The isolation and characterization of ubiquinone (coenzyme Q), ubichromenol, plastoquinone; chemistry, electron transport, and organic and biologic studies of coenzyme Q; oxidation of quinols by mitochondrial preparations; oxidative phosphorylation involving quinol phosphates; biosynthesis of ubiquinone series in animals and micro-organisms; biosynthesis of monobenzenoid quinones, terpenoid side-chains of quinones, ubiquinones, and coenzyme Q in the rat; diet-induced changes in ubiquinone; chemistry and function of vitamin K; oxidation-reduction reactions of ubiquinone in heart and kidney mitochondria; role of ubiquinone in the respiratory chain; histochemical studies of effects of coenzyme Q_{10} and menadione on oxidative enzymes in normal and neoplastic cells; and the role of plastoquinone in the electron transport system of photosynthesis; 453 pages; 82 illustrations; 1960. Little, Brown and Company, Boston, Mass. Price, $11.00.